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Light Scattering Studies of Solutions of the Bacterial Polysaccharide (XM6) Elaborated by *Enterobacter* (NCIB 11870)

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SUMMARY

Light scattering studies have been made on solutions of the sodium and tetra-methyl ammonium salts of the anionic heteropolysaccharide XM6. The polymers may be modelled as stiff wormlike chains. In dilute solution no conformational change was observed upon increasing the ionic strength. Models for gelation at higher polymer concentration are discussed. The measured mass per unit length of the polymer favours an ordered helical conformation and a gelation mechanism involving association and possibly crystallization of segments of the helical chains.

INTRODUCTION

XM6 is the code-name for the extracellular polysaccharide produced by the bacterium *Enterobacter* (NCIB 11870) (Nisbet *et al.*, 1984). The polymer is an anionic branched heteropolysaccharide (O'Neill *et al.*, 1986) with the chemical repeat unit shown in Fig. 1. When dispersed in water XM6 samples are viscous liquids. At polymer concentrations above a critical concentration, dependent upon the molecular weight of the polymer and the composition of the accompanying counterions, addition of sufficient concentration of electrolyte results in gelation (Nisbet *et al.*, 1984). The gels are thermally reversible, 'melt' over a narrow range of temperature, and the 'melting-point' increases with increasing ionic strength. 'Melting' is accompanied by a sharp change in optical rotation which has been attributed (Nisbet *et al.*, 1984) to a conformational transition for the polysaccharide from a low temperature

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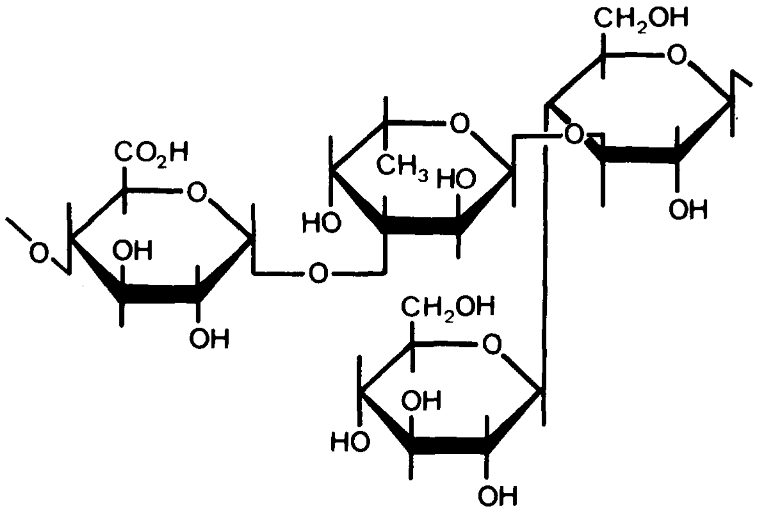

Fig. 1. Chemical repeat unit for XM6.

ordered conformation to a high temperature disordered ('random coil') structure. X-ray diffraction studies (Atkins, 1986; Atkins *et al.*, 1987*a*; Morris & Miles, 1986) of aligned fibres prepared from XM6 gels show highly crystalline patterns consistent with strong polymer–polymer association and gelation. Analysis of the X-ray data (Atkins *et al.*, 1987*a*), coupled with model-building calculations (Atkins *et al.*, 1987*b*), suggests an 8_3 double helix, sections of which can crystallize in either an orthorhombic or a tetragonal crystal modification. If the observed optical rotation transition (Nisbet *et al.*, 1984) is due to the melting of an eightfold double helix then it should be possible to detect such a transition, in the absence of interhelix association, by light scattering studies on dilute solutions of the polysaccharide. This article describes such studies under conditions favouring the proposed 'ordered' and 'disordered' states of the polysaccharide.

EXPERIMENTAL

Samples of XM6 were a gift from Dr I. T. Taylor (ICI plc, Biological Products Business, Billingham, UK). Large-scale production, isolation and clarification of XM6 polysaccharide was carried out as described by O'Neill *et al.* (1986). An additional clarification step involved filtering hot (90°C) aqueous dispersions through 8 μm Millipore filters. Pure salt forms of XM6 were prepared by ion-exchanging the polysaccharide to the acid form and then neutralizing solutions with the appropriate hydroxide.

Static light scattering studies were made using a modified Malvern 4300 spectrometer using 2·5 cm diameter scintillation vials as scattering cells. The light source was a vertically polarized He–Cd (wavelength $\lambda = 441{\cdot}6$ nm) laser. Instrument alignment was checked using filtered benzene samples and found to be satisfactory in the angular range 30–150°. The apparatus was calibrated with benzene. The Rayleigh ratio for benzene was calculated (Utiyama, 1972) to be $64{\cdot}23 \times 10^{-6}$ cm^{-1} for vertically polarized $\lambda = 441{\cdot}6$ nm radiation. Depolarization effects for XM6 samples were assumed to be negligible. Scattering data were collected at 25°C, over the angular range 30–150°, and analysed by the Zimm method (Zimm, 1948). Samples were cleaned by filtration through 3 μm Millipore filters. Clarification of samples and instrument calibration procedures were checked by studies on standard samples of polystyrene in toluene.

The refractive index of solutions was measured using an Abbé refractometer. Specific refractive index increments were determined at $\lambda = 632{\cdot}8$ nm using a Chromatix KMX-16 laser differential refractometer. The instrument was calibrated at 25°C using aqueous NaCl solutions. Polysaccharide solutions were equilibrated for ~30 min at 25°C before making measurements. Polysaccharide concentrations were determined by weighing freeze-dried and then vacuum-dried aliquots of known volume. The specific refractive index increment ($\mathrm{d}n/\mathrm{d}c$) was found to be 0·156 $\mathrm{cm}^3\,\mathrm{g}^{-1}$ ($\lambda = 632{\cdot}8$ nm). This value was corrected as described by Huglin (1972) to give a value of 0·157 $\mathrm{cm}^3\,\mathrm{g}^{-1}$ at $\lambda = 441{\cdot}6$ nm. Measurements of partial specific volume were made using an Anton Parr DMA 602 density measuring cell and DMA 60 meter.

Optical rotation measurements were made using an AA-100 polarimeter (Optical Activity Ltd).

RESULTS AND DISCUSSION

The optical rotation change accompanying the melting of a Na XM6 gel in the presence of added 0·5 M NaCl is shown in Fig. 2. Nisbet *et al.* (1984) reported that gelation was sensitive to the size of the counterion. For group I alkali metals optimum gelation was observed in the presence of sodium ions. The use of bulky cations such as tetra-methyl ammonium (TMA) ions should reduce intermolecular association and gelation. Addition of 0·5 M TMACl to Na XM6 or addition of 0·5 M NaCl to TMA XM6 both result in gelation. The optical rotation for TMA XM6 in the

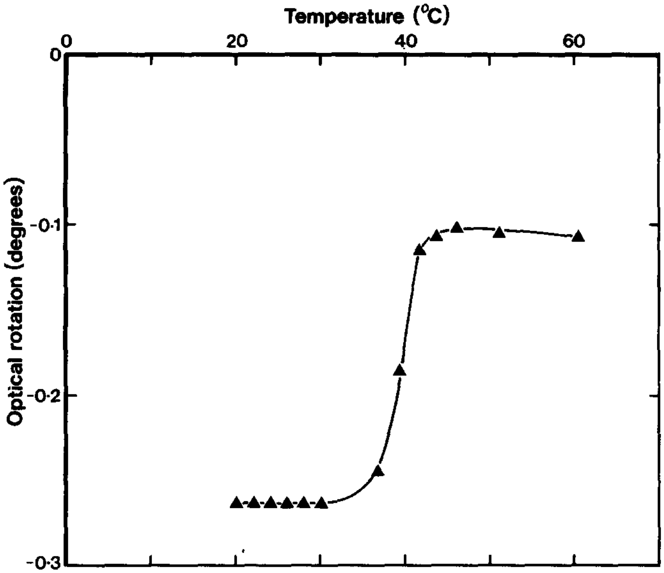

Fig. 2. Optical rotation change observed on melting a Na XM6 gel prepared in the presence of 0·5 M NaCl. Sample concentration 2% (w/w), cell length 10 cm, wavelength 589 nm.

absence of added electrolyte was found to be negative and the magnitude decreased monotonically with decreasing temperature (Fig. 3). TMA XM6 samples with added TMA Cl showed a similar optical rotation transition (Fig. 3) to that observed for the Na XM6 with 0·5 M NaCl (Fig. 2). In the case of TMA cations the transition temperature is lower and the samples were viscoelastic rather than true elastic gels. If the optical rotation transition monitors a conformational change then this transition may be observed in the presence of either sodium or tetra-methyl ammonium ions.

Static light scattering studies were made on Na XM6 and TMA XM6 in the absence of added electrolyte. These experimental conditions correspond to the proposed disordered ('random-coil') state of the polysaccharide. A Zimm plot for Na XM6 is shown in Fig. 4. Calculated values of the molecular weight $\langle M \rangle_W$, the radius of gyration $\langle Rg^2 \rangle_Z^{1/2}$, and the second osmotic virial coefficient A_2 for Na XM6 and TMA XM6 are listed in Table 1. The high molecular weights and high radii of gyration make the normal Zimm extrapolation difficult to carry out and the agreement of the results obtained for different ion forms of XM6 may be regarded as satisfactory. The stiffness of the polymer chains may be estimated by comparing the measured radii of gyration with the idealized value $(nl^2/6)^{1/2}$ expected for a freely jointed chain of n residues each of

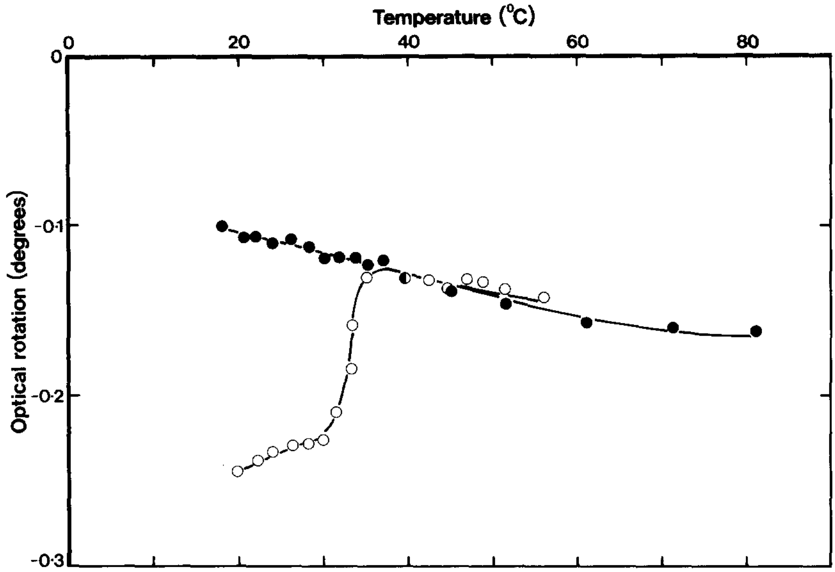

Fig. 3. Temperature dependence of the optical rotation for 2% (w/w) TMA XM6 (●) and 2% (w/w) TMA XM6 plus 0·5 M TMA Cl (○). Cell length 10 cm and wavelength 589 nm.

length l. For XM6 n may be taken to be the number of sugar residues in the polymer backbone. If M_S is the molecular weight of the chemical repeat unit then $n = 3\langle M\rangle_W/M_S$. Taking $M_S = 643$ daltons and $l = 0{\cdot}437$ nm gives idealized radii of gyration for Na XM6 and TMA XM6 of 17·2 and 16·3 nm respectively. Expansion of the polymer chain may arise in several ways.

Electrostatic repulsion between inadequately screened charged residues can lead to chain expansion. However, the charge density along the polymer chain is low and the slopes of the constant concentration curves in Fig. 4 do not increase with decreasing polymer concentration suggesting that the counterion screening is adequate at these polymer concentrations. Such changes in the angular dependence of the scattered light are reported by Oth and Doty (1952) in their studies of the dissociation of the carboxyl groups of polymethacrylic acid, under non-isoionic conditions. Segment–segment and segment–solvent interactions can swell the polymer chain and these can be estimated by calculating the swelling parameter (α) from the Flory–Orofino equation (Flory & Orofino, 1957)

$$A_2 = \frac{16\pi N_A}{3^{2/3}} \left\{ \frac{\langle Rg^2\rangle_Z^{3/2}}{\langle M\rangle_W^2} \right\} \ln \left\{ 1 + \left(\frac{\pi^{1/2}}{2} \right) (\alpha^2 - 1) \right\} \qquad (1)$$

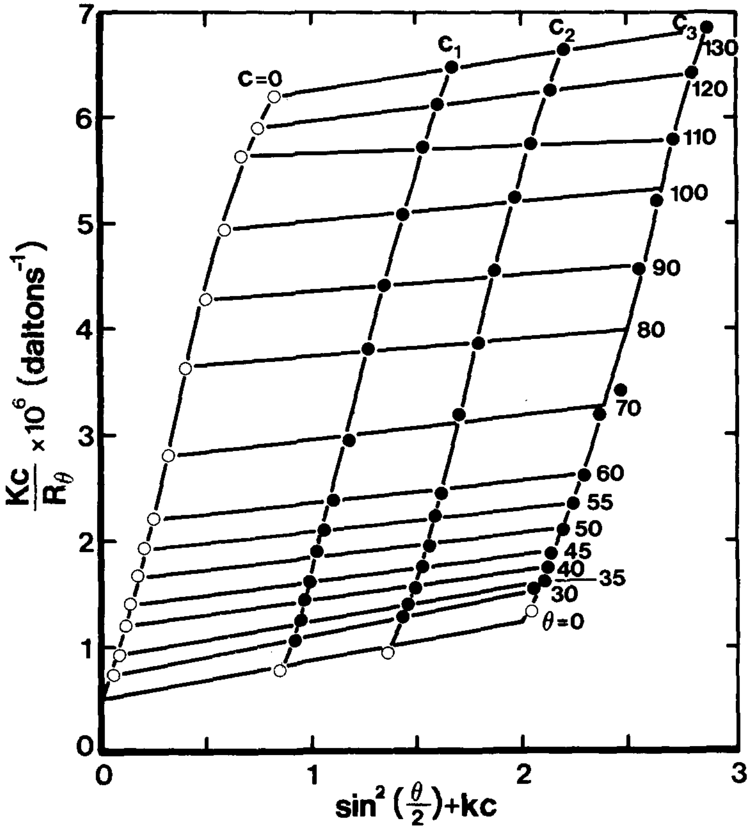

Fig. 4. Zimm plot for Na XM6 solutions. K is the scattering constant, R_θ the Rayleigh ratio, θ the scattering angle and c the sample concentration. $c_1 = 0{\cdot}55$ mg cm^{-3}, $c_2 = 0.89$ mg cm^{-3}, $c_3 = 1{\cdot}33$ mg cm^{-3}, $k = 1531$ cm^3 g^{-1}, wavelength = 441·6 nm.

where N_A is Avogadro's number. For both Na XM6 and TMA XM6 the values of $\alpha = 1{\cdot}006$ are very close to unity suggesting that the unperturbed dimensions of the molecules may be calculated directly from the light scattering data. For worm-like chain semi-rigid polymers the radius of gyration is dependent on the persistence length (a_0) and the contour length ($L = N_a a_0$) of the chain (Kratky & Porod, 1949)

$$Rg^2 = a_0^2 \left\{ \frac{L}{3a_0} - 1 + \frac{2a_0}{L} \left(1\text{-exp} \left(-\frac{L}{a_0} \right) \right) \right\} \tag{2}$$

For monodisperse polymers it is necessary to know the mass per unit length m_0 in order to calculate $L = M/m_0$ and hence a_0 from Rg^2. For the

TABLE 1
Light Scattering Data

Sample	$\langle M\rangle_W \times 10^{-6}$ (daltons)	$\langle Rg^2\rangle_Z^{1/2}$ (nm)	$A_2 \times 10^4$ ($\mathrm{cm^3\ mol\ g^{-2}}$)
Na XM6	2·0 ± 0·3	153 ± 7	1·3
TMA XM6	1·8 ± 0·3	160 ± 8	1·1
TMA XM6 + 0·5 M TMA Cl	1·4 ± 0·2	165 ± 8	1·3

disordered polyanion $m_0 \approx 490$ daltons nm^{-1} and this gives an estimated value of $a_0 = 18$ nm. This value is about twice the value observed for stiff cellulosic derivatives. The mixed linkages present in the chemical repeat unit (Fig. 1) suggest that the polymer is unlikely to be as expanded as the regular $\beta(1 \rightarrow 4)$ linked cellulose backbone. For polydisperse systems the value of a_0 calculated from $\langle Rg^2\rangle_Z$ and $\langle M\rangle_W$ using equation (2) will be too large. The effect of polydispersity can be estimated approximately by the following procedure. From equation (2) and the measured $\langle Rg^2\rangle_Z$ and $\langle M\rangle_W$ it is possible to use m_0 as a variable and plot a graph of a_0 vs m_0 (Fig. 5). To reduce $a_0 \lesssim 10$ nm it would be necessary to reduce m_0 to $m_0 \lesssim 280$ daltons nm^{-1} which is equivalent to putting $\langle M\rangle_Z / \langle M\rangle_W \approx \langle M\rangle_W / \langle M\rangle_n \gtrsim 1.8$. Thus a polydisperse solution of moderately stiff disordered coils could explain the measured value of $\langle Rg^2\rangle_Z$. To assess this model it would be necessary to determine the polydispersity or the mass per unit length. Determination of the molecular weight distribution is difficult but, under favourable experimental conditions, the mass per unit length of semi-rigid chains may be obtained from a Holtzer plot (Schmidt *et al.*, 1985) as recently demonstrated for the bacterial polysaccharide xanthan (Coviello *et al.*, 1986). A Holtzer plot of the 'zero-concentration' scattering data for Na XM6 is shown in Fig. 6. Since $qR_\theta/(\pi Kc)$ must tend to zero as $q\langle Rg^2\rangle_Z^{1/2}$ tends to zero the plot exhibits the shape expected for semi-flexible or worm-like chain molecules. It was not possible to clearly define the position of the peak at low $q\langle Rg^2\rangle_Z^{1/2}$ or the plateau value at high q. The limiting value of ~ 1400 daltons nm^{-1} is very high for a disordered coil and suggests some degree of molecular association.

Nisbet *et al.* (1984) noted a sharp change in optical rotation accompanying the melting of XM6 gels. These authors observed that the transition temperature was dependent upon ionic strength but independent of polymer concentration. A Zimm plot for TMA XM6 in the presence of 0·5 M TMA Cl is shown in Fig. 7 and the calculated values of $\langle M\rangle_W$,

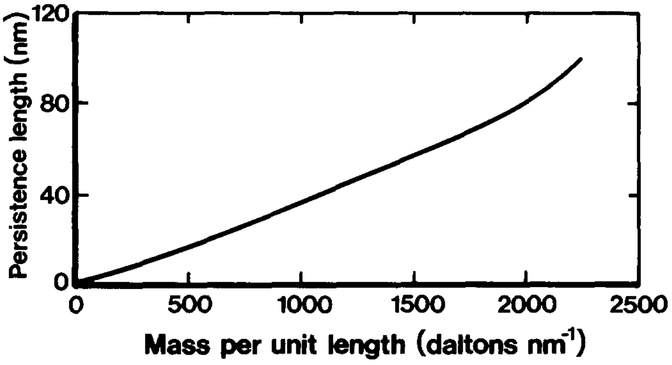

Fig. 5. Calculated plot of persistence length vs mass per unit length for Na XM6.

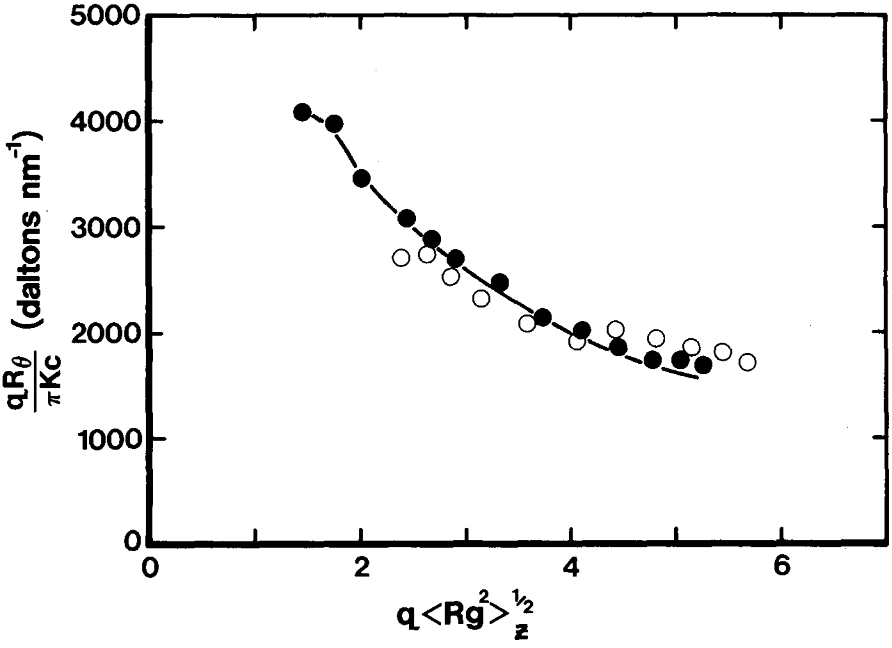

Fig. 6. Holtzer plots of the 'zero concentration' scattering data for Na XM6 (●) and TMA XM6 + 0·5 M TMA Cl (○) solutions. R_θ = Rayleigh ratio, K = scattering constant, $q = (4\pi n_0/\lambda)\sin(\theta/2)$ where n_0 is the refractive index of the solvent, λ the wavelength of the incident light and θ the scattering angle.

$\langle Rg^2\rangle_Z^{1/2}$ and A_Z are listed in Table 1. These experimental conditions correspond to the proposed ordered conformation. Upon raising the ionic strength to values favouring the proposed ordered conformation there is no observable increase in molecular weight or change in radius

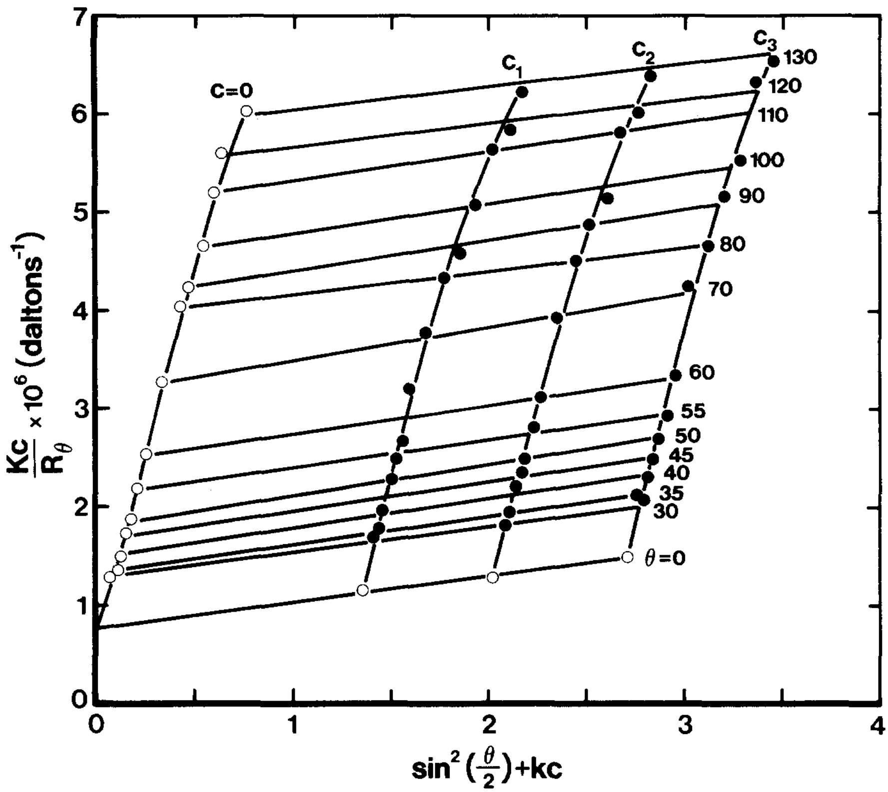

Fig. 7. Zimm plot for TMA XM6 solutions in 0·5 M TMA Cl. $c_1 = 1{\cdot}77$ mg cm^{-3}, $c_2 = 2{\cdot}49$ mg cm^{-3}, $c_3 = 3{\cdot}18$ mg cm^{-3}, $k = 842$ cm^3 g^{-1}, $\lambda = 441{\cdot}6$ nm.

of gyration. A Holtzer plot of the 'zero-concentration' scattering data for TMA XM6 in the presence of 0·5 M TMA Cl is shown in Fig. 6. The limiting value at high $q\langle Rg^2\rangle_Z^{1/2}$ suggests that there is no significant change in mass per unit length upon increasing the ionic strength. The present results suggest that the molecular conformation remains unchanged upon increasing the ionic strength. This suggests two alternative models for gelation depending on whether the molecular conformation is a disordered coil or an ordered helix. In model I (Fig. 8a) the polymer conformation is a disordered coil and helix formation only occurs upon aggregation and the formation of junction zones. In model II (Fig. 8b) the polymer adopts the double helical structure even at low ionic strength. Junction zone formation will involve helix–helix association. Both the persistence length and the mass per unit length will be different for a disordered coil and a double helix. The mass per unit length for the coil will be 490 daltons nm^{-1}. The X-ray data favours an 8_3 double helical struc-

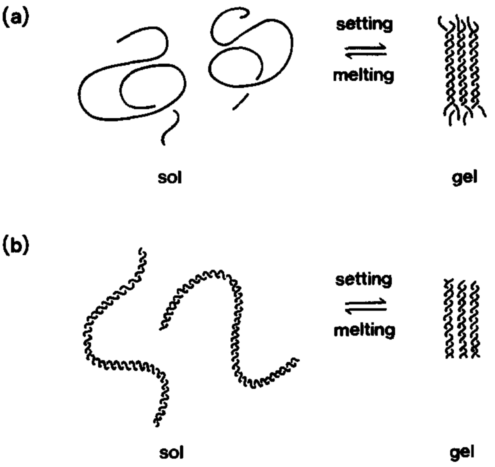

Fig. 8. Suggested models for the gelation of XM6 solutions. (a) Sol–gel transition involving a coil–helix transition and (b) sol–gel transition involving helix–helix association.

ture for the ordered conformation. The measured layer line spacing in the X-ray pattern was 4·95 nm suggesting a mass per unit length of ~ 1040 daltons nm^{-1} for the polyanion. This value is much closer to the limiting value observed in the Holtzer plots (Fig. 6) and the present experimental data favour the ordered conformation and model II (Fig. 8b). The presence of a few gel precursors would effectively increase the measured mass per unit length. For a double helix the persistence length estimated from Fig. 5 would be $a_0 \approx 39$ nm. The preparation of a series of molecular weight fractions and a study of the dependence of $\langle Rg^2\rangle_Z^{1/2}$ on $\langle M\rangle_W$ might provide an additional means of discriminating between models I and II.

In both the models illustrated in Fig. 8 the optical rotation change would only occur upon junction zone formation. There are at least two possible contributions to this change in optical rotation — namely a change in the backbone geometry of the polymer (coil–helix transition) and/or a change in the environment of the carboxyl group. The latter might be expected to dominate the optical rotation change for model II (Fig. 8b) whereas both should contribute to the change in optical rotation for model I (Fig. 8a). Nisbet *et al.* (1984) obtained circular dichroism data on XM6 samples. In the sol state there is a negative band at ~ 210 nm assigned to the carboxyl $n \rightarrow \pi^*$ transition of D-glucuronic acid. Upon cooling and gelation the molar ellipticity per glucuronate residue shows an appreciable enhancement in intensity which follows the observed temperature course for the change in optical rotation. It has been suggested that this change in circular dichroism may arise due to site binding of cations upon gelation and this suggestion (Nisbet *et al.*, 1984) is not inconsistent with the highly crystalline patterns observed in the X-ray diffraction studies of aligned XM6 gels (Atkins, 1986; Atkins *et al.*, 1987; Morris & Miles, 1986). A quantitative analysis of the contribution of the change in circular dichroism at 210 nm to the observed change in optical rotation at higher wavelengths may also provide a means of discriminating between models I and II (Fig. 8).

The present data suggest that the helical form of the polymer is stable at room temperature, even at low ionic strength, and that the sharp change in optical rotation accompanying melting of the gel is due to helix–helix association. Nisbet *et al.* (1984) note decreases in the broadening of resonances in the proton nmr spectra upon heating which are not coincident with the sharp change in optical rotation or the melting of the gel. The proton spectra obtained in the sol state shows anomeric resonances at 5·25 (glucuronic acid) and 5·4 (fucose). Thus there is some evidence for increased motion of at least a fraction of the backbone sugars. Therefore the possibility of a helix–coil transition *upon heating* requires further investigation.

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