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The influence of acetyl and pyruvate substituents on the helix–coil transition behaviour of xanthan

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ABSTRACT

Xanthans (Na^+ salt form) having various contents of acetyl and pyruvic acid groups were prepared by culturing different strains of *Xanthomonas campestris* and by deacetylation and depyruvation. Optical rotation ($[\alpha]_{365}$) was used to characterize the helix–coil transition behaviour of these polymers in deionized water. There were correlations between the acetyl and pyruvic acid contents and the mid-point temperature of the transition, between the pyruvic acid content and $[\alpha]_{365}$ in the high-temperature-plateau (coil) region of the curve, and between the content of pyruvic acid and the height of the transition. In deionized water, each of the polymers showed marked thermal hysteresis and a time-dependent fall in $[\alpha]_{365}$ at low temperatures. This behaviour, which was attributed to kinetic factors, was eliminated by the addition of NaCl. Salt also increased the melting temperature and reduced $[\alpha]_{365}$ in the low-temperature-plateau region of the curve in relation to the charge carried by the polymer. A high-pyruvate, low-acetyl xanthan exhibited unusual two-phase helix–coil transition behaviour in the presence of salt.

INTRODUCTION

Xanthan, the exocellular polysaccharide produced by *X. campestris*, is a (1→4)-β-D-glucan with β-D-Man-(1→4)-β-D-GlcA-(1→2)-α-D-Man side chains β-(1→3)-linked to alternate residues of the backbone. The internal mannosyl residue frequently carries an *O*-acetyl group at position 6 and the terminal mannosyl group may carry a 4,6-linked pyruvic acid acetal substituent[1]. The proportion of acetyl and pyruvate groups varies with the producing strain[2–4] and with the conditions of culture[5–7]. Values of up to 5% of acetyl and 8.1% of pyruvate are possible theoretically, assuming one acyl group per side chain, but the proportion of acetyl groups sometimes exceeds this value. The location of the additional acetyl groups is uncertain, but multiple acetylation of the internal mannosyl residue is possible[8] in some xanthans, as is acetylation of the terminal mannosyl group[9].

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X-Ray fibre diffraction studies[10] have indicated that the xanthan molecule adopts a right-handed helical conformation with five-fold symmetry and a pitch of 4.7 nm. The side chains were believed to fold back onto the backbone of the polymer, but attempted molecular modelling using potential energy calculations[11] has indicated that the side chains can adopt any of several possible conformations. Whether the polymer exists in solution as a single helix[12–14], a double helix[15], or a dimer formed by the association of two single helical chains is the subject of controversy, although double-chain models now seem to be gaining acceptance.

On heating, the xanthan molecule normally undergoes a reversible conformational transition from the ordered helical structure, believed to exist in solution, to a more flexible disordered state, frequently (though probably inaccurately) referred to as a random coil. The transition has been demonstrated by viscometry[12,16], optical rotation, circular dichroism, and relaxation n.m.r. spectroscopy[12]. The molecular basis for the transition is not understood. Morris *et al.*[12] envisaged an intramolecular transition in which the ordered single-helical structure was destabilised by disruption of the intramolecular non-covalent bonds, leading to the adoption of a "random coil" state. Milas and Rinaudo[13] concurred with this view. Liu and co-workers[15,17], on the other hand, proposed a model in which the double helix, upon heating, melts from both ends to form an expanded dimer comprising two molecules mainly in the random coil state but linked by a short region of double helix. Haache *et al.*[18] supported this view but suggested that, at higher temperatures, the dimer can dissociate to give disordered single strands.

The transition behaviour of xanthan is influenced by the ionic strength and pH. As the ionic strength (I) increases, so does the temperature of the mid-point of the transition (T_m)[12,13,17,19–21], and there is a linear relationship between T_m and log (I)[12,19]. Under conditions of low I, repulsion between the carboxylate groups tends to destabilize the structure, but, if salt is added, the cations stabilize the ordered conformation against disruption by heat. Divalent cations (Ca^{2+}) are more effective[19] at stabilizing the ordered structure than are monovalent ions (Na^+). In addition, Ca^{2+} reduces the range of temperature over which the transition occurs, whereas Na^+ does not[19]. Reducing the pH[19] and adding urea[22] also stabilize the ordered conformation. The reason for the effect of the latter is unknown but, by modifying the dielectric constant of the medium, urea may promote apolar interactions within the molecule.

The degree of substitution by acetyl and pyruvate groups affects the stability of the helix. Comparison of the mid-point temperature of the transition for xanthan with that of its deacetylated and/or depyruvated derivatives[20,23–25] has shown that removal of the acetyl groups produces a fall in the melting temperature, but that depyruvation results in an increase in T_m. Smith and co-workers[26] demonstrated that the melting temperature was dependent upon the ratio of pyruvate to acetyl groups, the ordered conformation becoming more stable as the proportion of acetyl groups increased or that of the pyruvic acid content decreased. The destabilizing effect of pyruvate can be explained by the increase in internal repulsion between the charged side chains. However, the reason for the stabilizing influence of the acetyl groups is less obvious. Apolar interactions between the acetyl methyl groups may be responsible[26] or, alternatively, the acetyl substituents could act as hydrogen bond acceptors, thereby stabilizing the molecule[24].

As well as affecting the melting temperature of xanthan, the distribution of acetyl and pyruvic acid groups may affect the range of temperature over which the transition occurs[20]. Holzwarth and Ogletree[20] demonstrated that the removal of pyruvic acid residues caused a significant sharpening of the helix–coil transition, whereas the removal of acetyl groups did not. The pyruvate content of the xanthan molecules within a population varies significantly[5,27,28], and the narrowing of the transition upon depyruvation was attributed to the switch from a heterogeneous to a more homogeneous population. Deacetylation had no effect because the distribution of acetyl groups within the native polymer was stoichiometric.

At constant ionic strength, the melting temperature of xanthan is unaffected by the concentration of the polysaccharide[12,13], but the mol. wt. appears to have some influence. The data in this area are contradictory. Liu and Norisuye[15], using fractions of different mol. wt. generated by sonication, produced evidence to suggest that, above 3×10^5, the helix–coil transition as monitored by $[\alpha]_{300}$ is unaffected by differences in mol. wt.; below 2×10^5, $[\alpha]_{300}$ shows a profound dependence on mol. wt. As the latter decreases, so does the melting temperature, and $[\alpha]_{300}$ in the low-temperature-plateau region of the curve becomes less negative, suggesting a reduction in the amount of order. Milas and Rinaudo[29], however, using xanthan fractions produced by partial hydrolysis with cellulase, showed that the melting temperature fell as the mol. wt. decreased for three samples of 3.2×10^6, 7×10^5, and 2×10^5. Two of these samples have mol. wts. above the threshold proposed by Liu and Norisuye[15], and no obvious explanation is available.

From reports in the literature, it is unclear whether or not solutions of xanthan show thermal hysteresis. Milas and Rinaudo[13] studied the helix–coil transition, by optical rotation, as a function of [NaCl] and found no hysteresis. Frangou and coworkers[22] showed that, in the presence of urea, the transition was also reversible. In contrast, Liu *et al.*[17] noted that, in 0.01M NaCl, there was significant thermal hysteresis, which diminished if the concentration of salt was increased. Liu and Norisuye[15] stated that, for any given temperature, solutions of xanthan quickly reached an equilibrium state. Holzwarth[19] observed that if an aqueous solution of xanthan was heated and then cooled quickly it retained the optical rotation of the denatured state. Even after storage for 45 days at 5°, the value of $[\alpha]_{365}$ returned only half-way towards that of the native conformation. The conflicting nature of these reports suggests that the behaviour of xanthan with respect to thermal hysteresis is influenced markedly by the environmental conditions.

The use of optical rotation data to characterize the helix–coil transition behaviour of native and chemically modified xanthans having widely different contents of acetyl and pyruvic acid groups is now reported.

EXPERIMENTAL

Materials. — Samples of xanthan were produced by culturing *X. campestris* strains *campestris* 646 (ATCC 13951), *phaseoli* 1128, and *phaseoli* 556 (both from the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts). Flocon 4800C was obtained from Pfizer Ltd.

Production and purification of xanthans. — The strains of bacteria were grown in batch (1 L) culture, using a sulphate-deficient medium[6]. The cultures were incubated for 4–5 days at 30° on an orbital shaker.

After fermentation, the cells were removed by centrifugation for 30 min at 10 000*g*. The supernatant solution was concentrated using a Pellicon cassette system (Millipore) with a polysulfone PTHK cassette of porosity 100 000 mol. wt., and the concentrate was treated with 2 vol. of cold acetone. The resulting precipitate was dissolved in distilled water, reprecipitated, and redissolved. It was then centrifuged for 75 min at 100 000*g* in order to remove subcellular debris, and dialysed against distilled water for 48 h at 4°. The polymer was converted into the Na^+ form by passage through Amberlite IR-120 (Na^+) and IRA-410 (Cl^-) resins, and the eluate was dialysed against distilled water and lyophilized. Flocon 4800C was resuspended in distilled water and similarly purified, starting at the ultracentrifugation step.

Modification of xanthan. — Xanthan was *O*-deacetylated by treating a 0.25% solution of the purified polymer with 0.1M NH_4OH for 1 h at 60°. The solution was dialysed against running tap water overnight and for a further 48 h against distilled water at 4°. The polymer was then converted into the Na^+ form, redialysed, and lyophilized.

Depyruvation involved treatment[30] of a 0.5% solution with 5mM trifluoroacetic acid at 100° for 90 min. The solution was then dialysed, passed through ion-exchange resins, redialysed, and lyophilized.

Chemical analysis. — Neutral sugars were determined[31] by h.p.l.c. at 85° on a Brownlee Polypore PB analytical cartridge (4.6 mm i.d. × 22 cm; Anachem Ltd.). They were eluted with deionized water at 0.2 $mL.min^{-1}$, and detected using a Knauer differential refractometer. Glucuronic acid was determined using the carbazole assay[32].

Acetyl and pyruvate groups were assayed by the hydroxamic acid[33] and 2,4-dinitrophenylhydrazine[34], methods respectively. All assays were performed at least in triplicate and the results are given as a percentage of the total carbohydrate, determined by the phenol–sulphuric acid assay[35].

Light scattering. — Static light-scattering measurements were carried out at the Institut für Makromolekulare Chemie (University of Freiburg, F.R.G.), using an ALV-Laser 3000 System with a servo-controlled SP-81 Goniometer. Sample preparation and data handling followed the procedures described by Coviello *et al.*[36].

Intrinsic viscosity. — The intrinsic viscosities were determined at 25°, in 20mM aqueous NaCl, using a Contraves Low Shear (LS30) couette viscometer. By measuring the ratio of the shear stress to an external shear rate ramp, the zero shear rate $[\eta]$ could be ascertained directly.

Polarimetry. — Optical rotation measurements were made at 365 nm using a Perkin–Elmer 241 polarimeter and 10-cm quartz cells. The temperature of the cells was controlled by a Haake F3 refrigerated water bath and the temperature of the samples was monitored with a chromel–alumel thermocouple.

Solutions 0.3% (w/w) of freeze-dried xanthan in deionized water were prepared by prolonged stirring, storage for 1 h at 90°, and then filtering through a 0.45-μm Millipore filter. Solutions containing NaCl were prepared by mixing equal volumes of filtered 0.6% xanthan and aqueous NaCl at double the required concentration. Mixtures were stirred at 90° in order to ensure homogeneity.

The sample solutions were loaded into the cells at 90° and, after degassing,were cooled at $1°.\mathrm{min}^{-1}$ to 10° and maintained thereat overnight (16 h). The course of the helix–coil transition was then monitored on the basis of changes in $[\alpha]_{365}$. Measurements were made in the range of 10–91°. The temperature was raised in increments of 3°, allowing 10 min at each step for equilibration. For cooling sweeps, the increments were as above, but the measurements were made directly after degassing.

Ion-exchange chromatography. — A solution of the polysaccharide (~ 10 mg) was applied to a column (22 × 2 cm i.d.) of DEAE-microgranular cellulose (Whatman DE52) and eluted with a gradient of 0→2M NaCl in phosphate buffer (pH 7.0) at ~ 10 $\mathrm{mL.h}^{-1}$. Fractions (3 mL) were collected and tested for carbohydrate. The appropriate fractions were combined, dialysed exhaustively against distilled water at 4°, reduced in volume using a rotary evaporator, and freeze-dried.

RESULTS

Chemical and physical analyses. — Data for the composition, $[\eta]$, and M_w of xanthans having different acetyl and pyruvic acid contents are given in Table I. Each sample had close to the expected 2:2:1 ratio of glucose, mannose, and glucuronic acid, indicating that no significant change other than loss of the substituent groups had occurred. Samples ps.646, ps.1128, and ps.556 had similar values for M_w, but the values for $[\eta]$ and the analysis of the dialysate after deacetylation and depyruvation indicated some reduction in the molecular weight of the materials. Depyruvation, in particular, may have caused significant depolymerisation.

Influence of the content of acetyl and pyruvic acid groups on the order–disorder transition. — The thermally induced changes in conformation were studied for each of the materials in the absence of salt. The heating and cooling curves were monitored, and measurements were performed, at least in duplicate, on freshly prepared samples.

An attempt was made to quantify the data for statistical analysis, and the following features of the transition curve were therefore measured: (*a*) transition midpoint, (*b*) range of temperature over which the transition occurred (transition breadth), (*c*) shift in $[\alpha]_{365}$ during the transition (transition height), (*d*) $[\alpha]_{365}$ at 15°, *i.e.*, in the low-temperature-plateau region of the curve, (*e*) $[\alpha]_{365}$ in the high-temperature-plateau region of the curve. The averaged data for each sample are given in Table II.

The parameters (*a*) to (*e*) were assessed by eye and, although there was a degree of

TABLE I

Chemical composition and intrinsic viscosity of xanthans

Xanthan[a]	*Glc:Man:GlcA*	*Ac* (%)	*Pyr* (%)	*Mol. wt.*	*Instrinsic viscosity (dl/g)*
ps.646	2.00:1.80:1.10	4.5	4.4	$0.9–1.2 \times 10^6$	34.1
DA ps.646	2.00:1.96:1.17	1.3	3.6	-	29.8
DP ps.646	2.00:1.82:1.13	4.4	0.6	-	15.5
DAP ps.646	2.00:1.84:1.17	1.0	0.6	-	14.4
ps.1128	2.00:1.82:1.06	7.7	1.7	1.27×10^6	79.5
DA ps.1128	2.00:2.10:1.17	1.6	1.3	-	24.5
ps.556	2.00:1.91:0.95	1.6	6.0	1.48×10^6	53.8
DP ps.556	2.00:1.65:0.85	1.1	1.0	-	16.4
Flocon 4800C	2.00:1.77:0.94	2.0	4.9	-	28.4
DP Flocon 4800C	2.00:1.71:0.88	2.7	1.0	-	10.5

[a] DA, deacetylated; DP, depyruvated; DAP, deacetylated and depyruvated.

subjectivity, the results were fairly reproducible. The melting temperature varied by not more than 1–2°. The transition breadth, the height of the transition, and $[\alpha]_{365}$ in the helix and coil plateaux regions of the curve usually varied more.

Some anomalous behaviour was observed. Sample ps.646 at <25° showed a large

TABLE II

The influence of acetyl and pyruvate groups on the helix–coil transition behaviour of xanthan

Polymer	*Ac (%)*	*Pyr (%)*	*Mean optical rotation data*				
			T_m (°)	*Transition breadth (°)*	*Transition height (mdeg)*	$[\alpha]^{15}_{365}$ *(mdeg)*	*Maximum* $[\alpha]_{365}$ *(mdeg)*
High Ac, High Pyr							
ps.646	4.5	4.4	44.0	23.5	40	–	−27
High Ac, Low Pyr							
DP ps.646	4.4	0.6	51.5	26.5	101	−109	+11
ps.1128	7.7	1.7	54.5	20.0	79	−126[a]	−21
Low Ac, High Pyr							
DA ps.646	1.3	3.6	31.5	32.5	86	−113	−25
ps.556	1.6	6.0	38.0	21.0	53	−37	+21
Flocon 4800C	2.0	4.9	27.5	22.0	37	−97	−60
Low Ac, Low Pyr							
DAP ps.646	1.0	0.6	40.0	23.5	127	−128	+21
DA ps.1128	1.6	1.3	42.0	29.5	158	−189	−24
DP ps.556	1.1	1.0	41.0	31.0	117	−73	+55
DP Flocon 4800C	2.7	1.0	45.0	24.5	120	−145	−10

[a] Determined by extrapolation of the baseline.

positive shift in $[\alpha]_{365}$ and gave erratic readings, which coincided with a fall in the transmittance of the sample and were probably due to aggregation. The pronounced tendency of ps.646 to aggregate in solution was demonstrated in light-scattering experiments[37]. Because of this phenomenon, it was not possible to be certain that the apparent levelling off of the transition curve at low temperature was genuine. Similar behaviour was observed intermittently for ps.1128, but the onset of the transition occurred at higher temperatures and the position of the baseline in the low-temperature-plateau region of the curve could therefore be established unequivocally. However, $[\alpha]_{365}^{15}$ had to be determined by extrapolation.

Sample ps.556 showed unusual transition behaviour in the presence of salt. What appeared to be a single conformational change in deionized water became two separate transition processes on the addition of salt. Hence, the data recorded for ps.556 in deionized water almost certainly describe a composite of two separate thermally induced transitions, which is therefore probably not comparable directly with that of the other polymers.

The effect of deacetylation and depyruvation upon the mid-point of the transition mirrors that described by others[20,23–25]. Deacetylation caused a decrease in the melting temperature, whereas depyruvation produced an increase. For ps.1128, a drop of 13° was observed, and for ps.646, a fall of 12.5°. Depyruvation of Flocon 4800C produced an increase in T_m of 17°, but the rise for ps.646 was much smaller (7.5°). Here, the stabilizing influence of the acetyl groups may have opposed the destabilizing affect of the pyruvic acid. A fall of only 3° was observed for ps.556 but, as already noted, this value may not be a genuine transition mid-point. The behaviour of ps.646 and its modified derivatives (Fig. 1) resembled that reported previously[23,24]. The depyruvated polymer had the highest melting temperature and this was followed in turn by the native xanthan, the deacetylated and depyruvated derivative, and the acetyl-free polymer. In Table II, where the polymers are grouped together according to their contents of acetyl and pyruvic acid groups, the behaviour follows essentially the same pattern.

The effects of the acetyl and pyruvate groups on other features of the transition curve are much less clear cut. Deacetylation caused a slight increase in the breadth of the transition for ps.1128 and ps.646. Samples ps.646 and Flocon 4800C were unaffected by depyruvation, but the transition breadth for ps.556 increased markedly. Any trends observed upon chemical modification, however, are not reflected by the groupings in Table II. Even omitting sample ps.556 from consideration, there is no discernible correlation between the breadth of the transition and the contents of acetyl and pyruvic acid groups.

Removal of the acetyl and pyruvate substituents caused a fall in $[\alpha]_{365}^{15}$ and an increase in the height of the transition; $[\alpha]_{365}$ in the high-temperature-plateau region of the curve was largely unaffected by deacetylation, but depyruvation caused a large positive shift (such a change can also be seen in the data of Holzwarth and Ogletree[20]). However, the averaged data for each sample in Table II show no consistent relationship between $[\alpha]_{365}$ for the polymer in the random coil state and the levels of substitution.

Detecting consistent patterns of behaviour within a set of data can be difficult

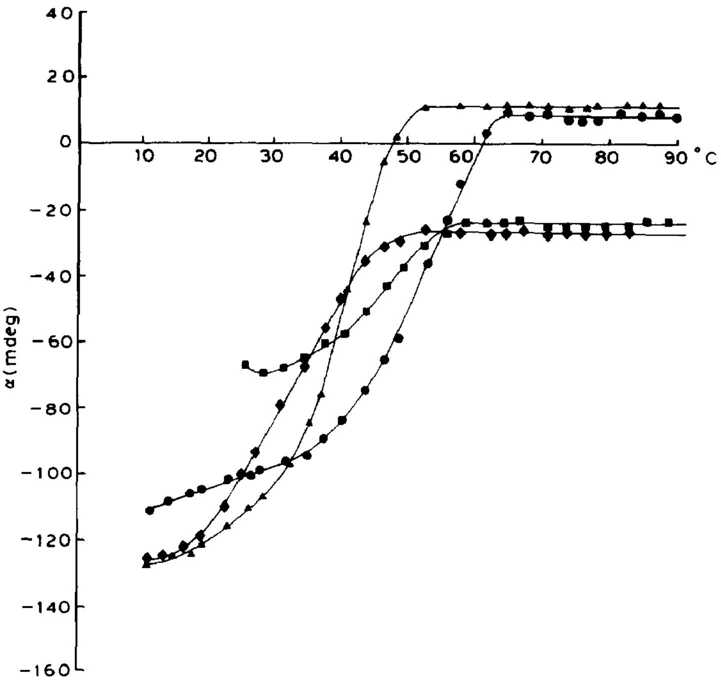

Fig. 1. Order–disorder transition for native and modified xanthan ps.646 (0.3%) in the absence of salt: ■, native; ◆, deacetylated; ●, depyruvated; ▲, deacetylated and depyruvated.

when more than one variable is involved. Apparent changes in the transition behaviour upon deacetylation and depyruvation often failed to hold good when a series of polymers with similar acetyl and pyruvic acid contents was considered, and variations in the data obtained for repeat experiments introduced additional difficulties. Hence, an attempt was made to assess the data statistically using a commercial computer package. The effects of the acetyl and pyruvic acid groups upon the transition were considered, together with the mol. wt. Measurements of the intrinsic viscosity in 20mM NaCl were used as an estimate of the relative mol.wts. of the samples in this study.

A multiple regression analysis of the data, using PROC RSQUARE from the SAS library[38], was carried out and the full correlation matrix of dependent and independent variables was computed. Model-dependent parameters, namely, melting temperature, the breadth and height of the transition, and the $[\alpha]_{365}$ values in the helix- and coil-plateau regions of the curve, were then regressed against the independent variables acetyl, pyruvate, and intrinsic viscosity. The independent parameters were tested singly, in pairs, and all together. The data for ps.556 and its depyruvated derivative were excluded because of the atypical behaviour of the native polymer. The data from the regression models for the dependent variables are shown in Table III. The $[\alpha]^{15}_{365}$ data were considered separately as no information was available for ps.646 (these data are bracketed). A value of 1 represents a perfect correlation, whereas zero indicates no correlation. R-square values of ≥ 0.5 are a satisfactory overall indication of statistical significance, for the number of samples tested.

TABLE III

R^2 correlations for single parameter, paired, and triplet regression models

Number in model	*Variables in model*	*R^2 for dependent variable*				
		T_m (°)	*Transition breadth (°)*	*Transition height (mdeg)*	*$[\alpha]^{15}_{365}$ (mdeg)*	*Maximum $[\alpha]_{365}$ (mdeg)*
1	% Ac	0.580	0.252	0.137	(0.026)	0.001
1	% Pyr	0.332	0.003	0.700	(0.225)	0.651
1	Intrinsic viscosity	0.124	0.139	0.129	(0.010)	0.107
2	% Ac % Pyr	0.904	0.256	0.844	(0.295)	0.652
2	% Ac Intrinsic viscosity	0.708	0.252	0.151	(0.027)	0.226
2	% Pyr Intrinsic viscosity	0.569	0.139	0.734	(0.225)	0.676
3	% Ac % Pyr Intrinsic viscosity	0.933	0.257	0.878	(0.458)	0.696

[a] The data for $[\alpha]^{15}_{365}$ are shown bracketed: this variable was considered separately as no information for ps.646 was available.

The correlations obtained reflect to a large extent the observations outlined above, but give them a more objective basis. The melting-temperature behaviour is of particular interest. An R-square value of 0.580 for acetyl alone increased to 0.904 when the two substituents were considered together, but inclusion of the intrinsic viscosity in the regression model barely improved the result. This finding indicates that the melting temperature of xanthan is determined by both the acetyl and pyruvic acid substituents, but mainly by the former.

No correlations were observed between the breadth of the transition and any of the independent variables, but a strong correlation was noted between pyruvic acid and $[\alpha]_{365}$ in the high-temperature-plateau region of the curve. This finding is consistent with the observed positive shift in $[\alpha]_{365}$ upon depyruvation. There was also a strong correlation between pyruvate and the height of the transition. This improved when acetyl and pyruvate were considered together, although there was no apparent correlation between acetyl and the height of the transition. The $[\alpha]^{15}_{365}$ values correlated with neither the acetyl nor the pyruvic acid content, which appears to conflict with the observed decrease in optical rotation on deacetylation and depyruvation but, in the absence of any data for ps.646, no high-acetyl and high-pyruvate polymer was considered.

There was no correlation between the intrinsic viscosity and any of the dependent variables, indicating that the transition behaviour of the polymers, as monitored by $[\alpha]_{365}$, is unaffected by molecular weight. Although $[\eta]$ may have been influenced by the presence of aggregates of high mol. wt. formed on freeze-drying (as occurs[40] for ps.1128), it should be a reasonably reliable estimate of the relative mol. wts. of the samples as used in the optical rotation experiments.

Thermal hysteresis and storage effects. — All of the polymers studied showed significant thermal hysteresis in the absence of salt. In addition, storage of the polymers without salt at 10°, overnight or for longer periods, resulted in a time-dependent fall in $[\alpha]_{365}$. Both phenomena were eradicated by the addition of NaCl.

Both the thermal hysteresis and storage effects can be explained in kinetic terms. When the rate of ordering is sufficiently fast in comparison with the rate of cooling, all of the conformational changes will have occurred during the cooling routine and hysteresis will not be observed. However, if the rate of ordering is much lower, then there is a time lag between the disordering and reordering of the molecules at any given temperature, and this is observed as hysteresis between the heating and cooling curves. Addition of salt promotes the ordering process and reduces the amount of hysteresis. This view is supported by the observation that, when the cooling regime in deionized water was halted in the middle of the coil→helix transition, $[\alpha]_{365}$ fell slowly during the succeeding hours, until it returned to the value obtained for the same solution, at the equivalent temperature on heating.

This type of kinetic behaviour would explain the discrepancies in the literature with respect to thermal hysteresis. Whether or not hysteresis is observed depends on how much salt is present in the sample and on the time and temperature regime over which the measurements are made. Thus, whilst Liu *et al.*[17] noted significant hysteresis even in the presence of salt, Milas and Rinaudo[13] may have failed to do so simply because the time course of their experiments was long enough to have allowed all of the conformational changes to occur.

Influence of salt upon the order–disorder transition. — The influence of salt upon the order–disorder transition was studied for samples ps.646, ps.1128, and ps.556. These polymers had very different acetyl and pyruvic acid contents; ps.646 was a typical wild-type xanthan with a substantial proportion of acetyl and pyruvic acid substituents, ps.1128 had a high content of acetyl, but very little pyruvate, and ps.556 was a high-pyruvate, low-acetyl xanthan. The transition behaviour of these polymers was studied in up to 30mM NaCl (Figs. 2–4).

The response of ps.646 and ps.1128 to salt was typical of xanthan. The optical rotation data indicated a single conformational transition on heating, which moved to higher temperatures as the concentration of salt increased. For ps.646, at >2mM NaCl, the mid-point of the helix–coil transition increased linearly with log [NaCl]. Insufficient data were available to establish whether this relationship held for ps.1128. Sample ps.556, however, behaved quite differently (Fig. 4). As already noted, the optical rotation curve for this polymer, upon addition of salt, was resolved into two separate transition steps, each of which moved to higher temperatures as the concentration of NaCl rose.

For each polymer, the position of the high-temperature-plateau region was unaffected by salt, but, in the low-temperature-plateau region, the baseline fell as the concentration of NaCl increased. The extent of the fall appeared to be related to the

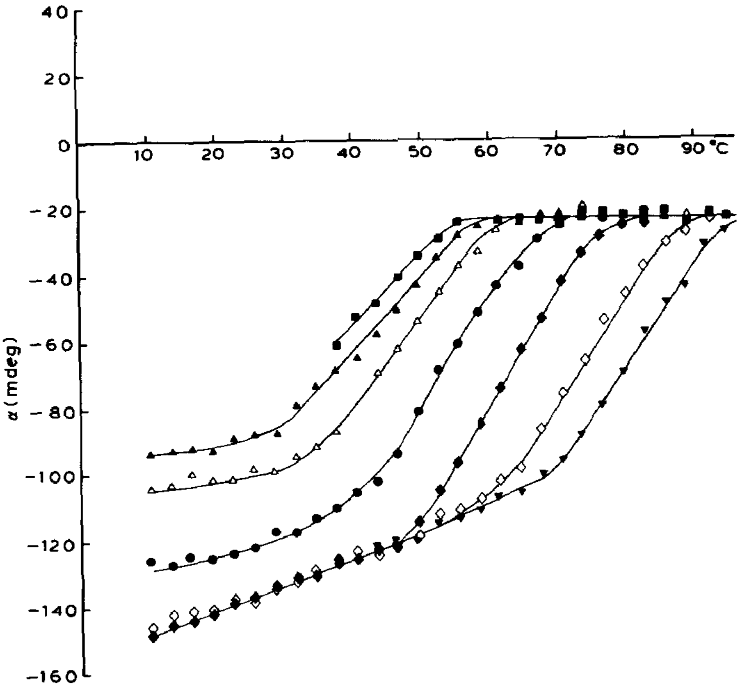

Fig. 2. Order–disorder transition for xanthan ps.646 (0.3%) in deionized water (■) and in 1 (▲), 2 (△), 5 (●), 10 (◆), 20 (◇), and 30mM (▼) NaCl.

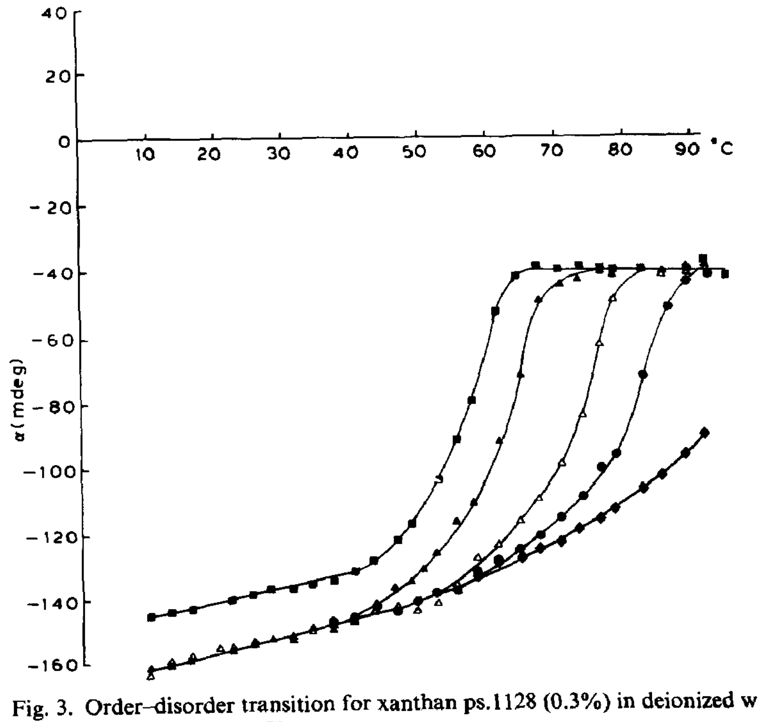

Fig. 3. Order–disorder transition for xanthan ps.1128 (0.3%) in deionized water (■) and in 1 (▲), 5 (△), 10 (●), and 30mM (◆) NaCl.

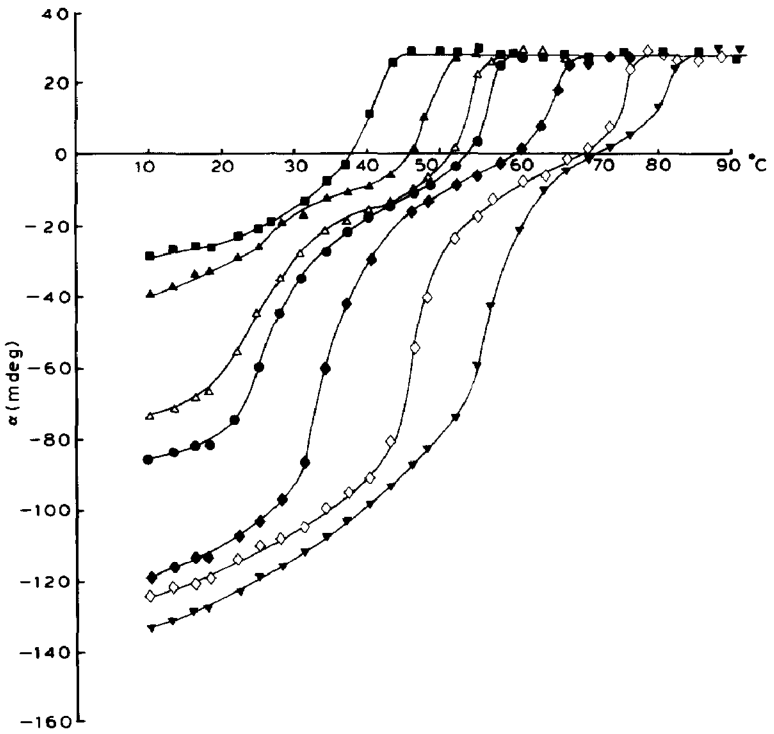

Fig. 4. Order–disorder transition for xanthan ps.556 (0.3%) in deionized water (■) and in 2 (▲), 4 (△), 5 (●), 10 (◆), 20 (◇), and 30mM (▼) NaCl.

amount of charge carried by the polymers. For the highly acetylated material, ps.1128, the fall in $[\alpha]^{15}_{365}$ was only 16 mdeg and the minimum, which corresponded presumably to the maximum degree of attainable order, was reached at mM salt. In contrast, for ps.556 with its high content of pyruvic acid, the fall in $[\alpha]^{15}_{365}$ was 102 mdeg and the baseline was still falling at > 20mM NaCl. Sample ps.646 showed intermediate behaviour. A fall of 51 mdeg was recorded between mM salt and the minimum attainable optical rotation, and the maximum level of order was achieved at 5–10mM salt. Dentini *et al.*[24] similarly noted that the optical rotation of an acetyl-free (*i.e.*, high-pyruvate) xanthan in the ordered state was markedly dependent upon the level of calcium perchlorate, but that, for native xanthan, depyruvated xanthan, and deacetylated and depyruvated xanthan, the effect was negligible. Interestingly, the minimum optical rotation was much the same for all of the polymers: $[\alpha]^{15}_{365}$ was -159 mdeg for ps.1128, -144 mdeg for ps.646, and -130 mdeg for ps.556. This suggests that, in the presence of sufficient salt, each polymer adopted a comparable degree of conformational order.

The transition breadth for ps.646 remained unchanged as the concentration of NaCl increased. The same behaviour was observed by Holzwarth[19] for Keltrol, a commercial polymer. The pattern of behaviour for ps.1128 was difficult to judge because of the small number of complete transition curves and the difficulty in discerning the precise temperature for the onset of the change in conformation.

The molecular basis for the two-step transition behaviour observed for ps.556 in the presence of salt is obscure. A two-stage change in conformation seems unlikely. A more probable explanation would be the existence of two well-defined chemical fractions within the sample, each of which undergoes the usual helix–coil transition but over a different range of temperatures. The level of pyruvate substitution can vary within a population of xanthan molecules[27,28] and fractions having high and low contents of pyruvic acid may be present in ps.556. The high-pyruvate fraction would undergo the low-temperature transition, and the low-pyruvate fraction that at higher temperatures. The pyruvic acid content of ps.556 was 6.0% and the theoretical maximum, assuming one pyruvate group per side chain, is 8.1%. Thus, if the high-pyruvate fraction were fully substituted, the non-substituted polymer could only account for ~25% of the total. This situation is consistent with the relative height of the transitions in the optical rotation scan. At high concentrations of salt, the height of the transition for the low-temperature change in conformation was about three times that of the high-temperature transition. Furthermore, the fall in the low-temperature-plateau region was observed in what would correspond to the high pyruvate fraction.

An attempt to fractionate ps.556 using ion-exchange chromatography yielded two polymer fractions of appropriate size. However, each contained almost the same amount of pyruvic acid as the unfractionated sample. A satisfactory alternative explanation is not readily available. Different charge distributions within the molecules seem unlikely since, with 6% total substitution, there is little scope for different arrangements of the side-chain pyruvic acid residues. The presence of xanthan fractions of high and low mol. wt. also seems improbable since, according to Liu and Norisuye[15], for the mol. wt. to have such a profound effect upon the transition behaviour, it would have to be well below 2×10^5 for one fraction at least. The weight-average mol. wt. of ps.556, determined by light scattering, was 1.48×10^6. The existence of two well defined fractions with such different mol. wts. seems unlikely, and it is doubtful whether the difference in mol. wt. would account for their separation by ion-exchange chromatography.

DISCUSSION

In line with the reports in the literature, an extremely good correlation was found between the melting temperature of the polymers and their contents of acetyl and pyruvic acid groups. Acetyl groups had a stabilizing effect upon the ordered conformation and raised the mid-point of the transition, whereas pyruvate substituents destabilized the structure and lowered the melting temperature. The destabilizing influence of the pyruvate substituents was the result of charge–charge repulsion between the trisaccharide side-chains, but the reason for the stabilizing influence of the acetyl groups is less certain.

Sodium chloride, by charge shielding, repressed intramolecular repulsion and stabilized the ordered conformation to a higher temperature. The amount of salt necessary to achieve the maximum attainable level of order increased with the amount of charge carried by the polymer. Thus, the fall in $[\alpha]_{365}^{15}$ was much greater for samples ps.646 and ps.556 than for ps.1128, and required a much higher concentration of added salt.

The effects of the acetyl and pyruvate substituents upon the breadth of the transition were not clear cut. Chemical deacetylation appeared to cause a slight increase in the range of temperature over which the transition occurred, whereas depyruvation produced no change, but no correlation could be found between the breadth of the transition and the contents of acetyl and pyruvate groups.

In theory, there are two ways in which the substituents could affect the breadth of the helix–coil transition. They could alter the co-operativity of the transition process or, as suggested by Holzwarth and Ogletree[20], they could affect it by virtue of their distribution within the population of molecules. The observed transition behaviour is difficult to explain in terms of either theory. One possibility would be that because acetyl stabilizes the ordered conformation to high temperatures when sufficient energy is supplied to disrupt the helix, subsequent conversion into the disordered state is rapid (*i.e.*, more cooperative). This possibility would be consistent with the increase in the breadth of the transition observed on deacetylation, but it is difficult to equate with an equilibrium model for the transition. The acetyl content of ps.646 was almost stoichiometric, whilst ps.1128 carried slightly more than one acetyl group per side chain. Nothing is known about the distribution of the acetyl groups but, from the quantities alone, deacetylation should produce either an increase in homogeneity or very little change. Neither is consistent with a broadening of the optical rotation curve.

Pyruvate, by destabilizing the ordered structure, might be expected to increase the co-operativity of the helix–coil transition. If this were so, then depyruvation should increase the breadth of the transition, but no such change was observed. In both ps.646 and Flocon 4800C, roughly one in every two side chains was substituted with pyruvic acid. If these substituents were distributed evenly, then depyruvation should produce no change in heterogeneity and therefore no change in the breadth of the transition. However, for ps.646, Sutherland[28] has demonstrated the existence of fractions with high and low contents of pyruvate. For this polymer at least, therefore, an increase in homogeneity and a narrowing of the breadth of the transition would be expected upon depyruvation if the argument of Holzwarth and Ogletree[20] applied.

It seems that neither the acetyl nor the pyruvic acid substituents played an important role in determining the breadth of the transition *via* either of the mechanisms outlined above, and this view is supported by the lack of correlation found between the acetyl and pyruvate content of the polymers and the breadth of the transition upon computer analysis.

There appeared to be a correlation between the height of the transition and the content of acetyl and pyruvate substituent; the lower the amount of each substituent, the greater the shift in optical rotation during the transition. The most obvious factor affecting the transition height would be the concentration of pentasaccharide repeating-unit. Since all of the measurements were made on 0.3% solutions of polysaccharide, those polymers with less acetyl and pyruvate will have contained more pentasaccharide as a proportion of the total weight. According to Rees[41], the optical rotation is related directly to the bond angles of the glycosidic linkages and the span of optical rotation therefore would be expected to increase upon removal of the acetyl and pyruvic acid substituents, as indeed it did. However, the change in the height of the transition was proportionally much greater. Other factors affecting the height of the transition would be the degree of order attained by the polymer at low temperatures, under the prevailing ionic conditions, and possible inhibition of ordering by ultrastructural features such as the absence of side chains from regions of the cellulosic backbone[42]. The height of the transition may also have been influenced by differences in the time-dependent fall in the low-temperature specific optical rotation prior to the experiment, and by the behaviour in the high-temperature-plateau region.

The reason for the fall in the baseline on cold storage is uncertain. It may have been due to kinetic factors, as already discussed, or to the aggregation of molecules in the ordered state. If regions of order and disorder can coexist within the same molecule, as they appear to do, then aggregation of the partially ordered strands could draw more of the molecule into the ordered form, thereby reducing the optical rotation. Under these conditions (low temperature, but medium-to-low ionic strength), the position of the equilibrium would then be:

$$\text{COIL} \rightleftharpoons \text{HELIX} \rightleftharpoons \text{AGGREGATION OF HELICES}$$

Such an argument requires that order is essential to aggregation. Therefore, the most highly ordered polymers should be the most aggregated and *vice versa*. Clearly, this situation was not true for ps.646 which showed a pronounced tendency to aggregate but had a relatively high $[\alpha]_{365}$ in the low-temperature-plateau region of the curve.

The behaviour of $[\alpha]_{365}$ in the high-temperature-plateau region of the curve was surprising. Assuming that Rees[41] is correct, $[\alpha]_{365}$ for the random coil state should have been much the same for all of the materials tested, and deacetylation and depyruvation should have had little effect. However, for those samples containing very little acetyl or pyruvate, the mean $[\alpha]_{365}$ varied from -24 to $+55$ mdeg, a span greater than the total height of the transition for some polymers. Deacetylation produced no change in $[\alpha]_{365}$, but depyruvation always produced a large positive shift. Indeed, there was a significant correlation between the amount of pyruvate and the maximum $[\alpha]_{365}$, which was strengthened when pyruvate and acetyl were considered together. Although the disordered state of xanthan is normally referred to as a "random coil", the available evidence, albeit limited, suggests that xanthan in the disordered state is still a relatively stiff molecule. Such evidence includes the relatively small change in intrinsic viscosity over the temperature range of the transition[13]. If xanthan in the high-temperature-plateau region of the curve is not genuinely a random coil, then the local geometry may well be influenced heavily by such structural features as the content of acetyl and pyruvic acid substituents.

In view of the poor correlations obtained, it might be thought that the differences in the breadth and height of the transition and $[\alpha]_{365}^{15}$ could be due entirely to kinetic factors. However, the parameters compared were measured only under heating conditions, and the equilibration time was certainly shorter than the 10 min allowed.

Attempts at explaining changes in $[\alpha]_{365}$ for xanthan have so far centred on the behaviour of the individual molecules in solution and any possible concentration effects have been ignored. Nevertheless, for all of the polymers studied, a 0.3% solution of the polysaccharide was well above the concentration c^* ($= 1([\eta])$ for overlap of chains and some effect on the optical rotation would be expected, and could perhaps account for certain of the anomalies observed. For example, the degree of overlap of chains could alter the equilibria above, and so influence the adoption of the ordered conformation. This would result in a time-dependent change in the low-temperature $[\alpha]_{365}$. Alternatively, the intermolecular interactions might affect the optical rotation directly. The relationship demonstrated by Rees[41] between the optical rotation and the glycosidic bond angles was semi-empirical, and apparently no attempt has yet been made to derive this relationship from first principles. Therefore, other factors besides the bond angles might influence the chiroptical behaviour, giving the wide variation in $[\alpha]_{365}$ observed at high temperatures. Hjelm *et al.*[43] showed that the c.d. spectrum in the soret region for fibre gels of sickle-cell deoxyhaemoglobin was affected by the linear dichroism and birefringence of the individual molecules, even when no net linear dichroism or birefringence could be demonstrated. Xanthan, which shows molecular anisotropy, could be affected similarly and the optical rotation altered accordingly.

Thus, it seems likely that much of the transition behaviour of xanthan cannot be explained simply, but may be influenced by a series of factors. Conclusions in the past have often been based on a somewhat restricted number of samples and experimental conditions, and this may account for some rather simplistic explanations for the behaviour of what is a very complex system.

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