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Influence of the Acetyl Substituent on the Interaction of Xanthan with Plant Polysaccharides — I. Xanthan–Locust Bean Gum Systems

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ABSTRACT

A range of xanthans (Na⁺ salt form) with varying levels of acetyl and pyruvic acid substitution were prepared by culturing different strains of Xanthomonas campestris *and by chemical deacetylation and depyruvylation. Oscillatory-shear measurements were used to characterize the interaction between these polymers and locust bean gum (LBG) in de-ionized water and the data were analysed statistically. The majority of the polymers interacted to form a strong thermoreversible-gel network, and the strength of the system was shown to be heavily dependent on the level of acetyl substitution. A polymer from a mutant strain of* X. campestris, *believed to lack the terminal mannose residue from the trisaccharide side-chains, formed an exceptionally weak gel network, suggesting that the xanthan side-chain may play an important role in the interaction with LBG.*

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INTRODUCTION

Xanthan is an exocellular polysaccharide slime produced by bacteria of the *Xanthomonas campestris* group. It consists of a β-(1→4)-D-glucose backbone with trisaccharide side-chains (1→3)-linked to alternate backbone residues. The side-chains are β-D-mannose-(1→4)-β-D-glucuronic acid-(1→2)-α-D-mannose. An *O*-acetyl group is frequently present at the C-6 position of the internal mannose residue, whilst the terminal mannose may carry a 4,6-linked pyruvic acid (strictly, pyruvate ketal) substituent (Jansson *et al.*, 1975). The proportion of the side-chains substituted varies with both the producing strain and the conditions under which the organism is cultured (Cadmus *et al.*, 1978; Davidson, 1978; Tait *et al.*, 1986).

X-ray fibre-diffraction studies have indicated that, in the solid state, the xanthan molecule adopts a right-handed helical conformation with fivefold symmetry and a helix pitch of 4·7 nm (Moorhouse *et al.*, 1977), and it is generally assumed that the same conformation exists in solution. Whether xanthan exists as a single helix, a double helix or as a dimer formed by the association of two single helical chains has been a matter of controversy, but double-chain models now seem to be growing in acceptance.

It has been known for some years that xanthan interacts with a range of β-(1→4)-linked plant glycans. These include certain glucomannans and galactomannans and even cellulose itself when first solubilized by derivatization. The Xanthomonads are plant pathogens and, since many of the polymers with which xanthan interacts are located within the plant cell wall, it has been suggested that such an interaction may be important in the initial adhesion and colonization of the host by the invading organism (Morris *et al.*, 1977).

The series of three papers, of which this part is the first, is concerned with the 'synergistic' interaction between xanthan and three plant polysaccharides, namely locust bean gum, guar gum and konjac mannan. The work sets out to characterize in rheological terms the interactions between the polymers in solution and to try to assess the influence of the xanthan acyl substituents.

Locust bean gum (LBG) is a galactomannan obtained from the seeds of *Ceratonia siliqua*. It consists of a β-(1→4)-D-mannan backbone with single α-(1→6)-linked D-galactose side-chains. The polymer typically contains 21–23% galactose, and the side-chains are distributed in a non-regular fashion with a high proportion of substituted couplets, lesser amounts of triplets and an absence of block substitution (McCleary *et al.*, 1985). Neither xanthan nor LBG will normally gel alone, but at high

enough concentrations (typically greater than 0·5% total polysaccharide) they interact to form a firm, rubbery thermoreversible gel network. The gels show sharp melting and setting behaviour. The melting temperature increases with the total polysaccharide concentration, but shows little dependence upon the relative concentrations of the two polymers (Dea *et al.*, 1977). Maximum gel strength occurs over a pH range of 6–8, and there is a dramatic decrease in gel strength at both the acid and alkaline ends of the pH spectrum (Kovacs, 1973). The optimum ratio of xanthan:LBG is uncertain — Kovacs has reported an optimum ratio of 1:1, Tako *et al.* (1984) a ratio of 1:2 and Dea and Morrison (1975) a ratio of 1:3. However, at least for 'low' molecular weights, the ratio would be expected to vary with the molecular weight of the polymers, so there may be no inconsistency.

Gelation between xanthan and LBG is generally believed to occur via the formation of mixed junction zones, although the precise nature of these junction zones is a matter of conjecture. At present, three different models are in contention.

Model 1 — Dea and co-workers (1977, 1986) demonstrated that the interaction between xanthan and the galactomannans is heavily dependent on the amount of galactose present and its distribution, and suggested a synergistic interaction between the xanthan helix and the unsubstituted or poorly substituted regions of the galactomannan. Moorhouse *et al.* (1977) observed that the 5/1 helical conformation favoured for xanthan presents two distinct faces, one with the side-chains and charged groups and the other comprising essentially the cellulosic backbone. It was suggested, therefore, that the interaction with the galactomannan might take place at the cellulosic 'groove', i.e. between the similar β-(1→4)-linked cellulosic and mannan backbones.

Model 2 — Rheological evidence obtained by Tako *et al.* (1984) apparently indicates the involvement of the xanthan side-chains in xanthan–galactomannan binding, and it was suggested that the side-chains of the xanthan in the helical conformation are inserted into adjacent unsubstituted regions of the galactomannan backbone. The galactomannan, it was proposed, is extended in a twofold, ribbon-like conformation, which produces a lock-and-key effect. Measurements in the presence of urea showed a marked fall in the 'dynamic viscoelasticity' of the mixed systems, suggesting that hydrogen bonding plays a major role in the interaction.

Model 3 — X-ray fibre diffraction is the only method presently available for studying gels at the level of atomic resolution, and even then the gel sample has to be stretched and at least partially dried to form a

fibre, so that it may no longer be in its 'native' state. This technique has been used by Cairns and co-workers to study the interaction between xanthan and LBG (Cairns *et al.*, 1986, 1987). The diffraction pattern for the xanthan–galactomannan system showed reflections characteristic of both aligned xanthan helices and mixed junction zones. However, gelation only appeared to occur if the xanthan was first denatured by heating. These workers noted that glucose and mannose differ only in the orientation of the —OH group at the C-2 position, and proposed therefore that binding occurs between the sterically compatible cellulosic backbone of xanthan in the disordered state, and the mannan backbone of LBG. The remaining xanthan, it was suggested, re-adopts the ordered, helical conformation.

The precise structure of the xanthan–galactomannan junction zones cannot be discerned from the X-ray fibre-diffraction data, but possible models have been suggested based upon the available information. According to Cairns *et al.*, the reflections for the mixed junction zones indicate a possible sandwich structure in which the xanthan side-chains are staggered. Staggering is suggested by the repeat-unit distance of 0·52 nm. (Other simpler binding schemes would be expected to give an axial advance per repeat unit of 1·04 nm.) The exact stoichiometry of binding is unknown. Several galactomannan molecules may be sandwiched between the xanthan backbones, and the interior of the sandwich might need to accommodate galactose substituted regions of the galacto-mannan backbone as well as the bare mannan regions. There is also evidence to suggest that growth of the sandwich structure can occur in two dimensions only. Additional support for this type of model has recently been presented by Cheetham and Mashimba (1988).

One alternative mechanism which has been largely neglected in favour of junction zone models is that of mutual incompatibility (as opposed to mutual compatibility) between different polymer species. In the absence of favourable polymer-1/polymer-2 interactions, the thermodynamic drive for mutual incompatibility at higher concentrations suggests that phase-separated networks are a likely result. Such mixed networks have been observed for a number of protein–polysaccharide systems (Brownsey & Morris, 1988). Although no direct evidence has been obtained for gross phase separation in xanthan–galactomannan systems, LBG can be induced to gel on its own at high effective concentrations, notably as a result of freeze-thawing or lowering of the water activity by addition of low-molecular-weight species such as sucrose and glycerol (Dea *et al.*, 1977, 1986). Mutual self-compatibility of the two components of a mixture produces 'phase domains' rich in each component

at effective concentrations above that required for gelation of the individual species. Generally, these domains are larger than the wavelength of light so that phase-separated gels are turbid, but the actual domain size will depend subtly on the balance of mutual and self interactions.

The importance of the galactose side-chains in xanthan–galactomannan binding is well established, but the role of the xanthan side-chains and in particular the acetyl and pyruvic acid substituents that they carry is as yet poorly understood. Both Dea *et al.* (1977) and Tako *et al.* (1984) have reported a stronger interaction between deacetylated xanthan and LBG than with the native polymer, and Tako *et al.* suggested that an increase in the flexibility of the xanthan molecule upon deacetylation could facilitate easier association between the xanthan side-chains and galactomannan backbone, giving rise to a stronger gelling interaction. Moreover, the evidence for this increase in flexibility is itself not very convincing (Shatwell *et al.*, 1990*a*). In contrast, Cheetham and Mashimba (1988) found that in terms of gel melting temperature (which they equated to gel strength), the gel formed by deacetylated xanthan and LBG after heating and cooling was similar to that formed by the parent polymer. The interaction was, however, significantly stronger than that of depyruvylated xanthan with LBG.

To date, there is very little evidence to suggest that pyruvic acid affects the strength of the gelling interaction. Cheetham and Mashimba (1988) found that a depyruvylated sample of commercial xanthan (Kelco Inc., San Diego, CA) failed to gel with LBG in distilled water, although it interacted quite strongly in the presence of 0·05 M KCl. This suggests that pyruvate may in fact promote gelation, at least under conditions of low ionic strength.

In this study, the interaction of LBG with a range of xanthans with different levels of acetyl and pyruvic acid substitution has been compared using oscillatory-shear measurements and minimum gelling concentration experiments. A polymer believed to lack the terminal mannose residue from the trisaccharide side-chain has also been studied.

EXPERIMENTAL

Materials

Xanthan samples

Samples of xanthan were produced by culturing the following bacterial strains: *X. campestris* pv. *campestris* 646 (ATCC 13951), *X. campestris*

pv. *campestris* BD9A (Tait & Sutherland, 1989), *X. campestris* pv. *phaseoli* 1128 and *X. campestris* pv. *phaseoli* 556 (both from the National Collection of Plant Pathogenic Bacteria, Harpenden, Herts, UK).

Production and purification

The bacterial strains were grown in batch culture using sulphate-deficient medium (Davidson, 1978). The medium was dispensed in 1 litre amounts into 2 litre Erlenmeyer flasks and cultures were incubated for 4–5 days at 30°C on an orbital shaker.

After fermentation, cells were removed by centrifugation for 30 min at 10 000 *g*. The supernatant was then concentrated using a Pellicon cassette system (Millipore) with a polysulfone PTHK cassette of porosity 100 000 molecular weight. The concentrate was treated with two volumes of cold acetone to precipitate the polymer, and the material was then redissolved in distilled water and precipitated once again. After resuspending for the second time, the polymer solution was ultra-centrifuged for 75 min at 100 000 *g* to remove subcellular debris, and dialysed against distilled water for 48 h at 4°C. Conversion to the sodium form was achieved by passing the material through sodium (Amberlite, IR-120) and chloride (Amberlite, IRA-410) ion-exchange resins. The polymer solution was then finally redialysed against distilled water to remove the sodium azide used as a preservative, and lyophilized.

Chemical modification

Deacetylation of xanthan was achieved by treating a 0·25% solution of the purified polymer with 0·1 M ammonium hydroxide at 60°C for 1 h. The solution was then dialysed against running tap water overnight and for a further 48 h against distilled water at 4°C. It was then converted to the sodium form, redialysed and lyophilized.

Depyruvylation was by treatment of a 0·5% polysaccharide solution with 5 mM trifluoroacetic acid at 100°C for 90 min (Bradshaw *et al.*, 1983). The solution was subsequently dialysed, passed through ion-exchange resins and lyophilized as for the deacetylated material.

Locust bean gum

LBG was prepared from a commercial flour using a modification of the method of McCleary *et al.* (1983). Crude galactomannan (15 g) was treated with 200 ml of boiling, aqueous 80% ethanol for 10 min. The slurry was collected on sintered glass and washed successively with ethanol, acetone and ether. This material was added to 1 litre of hot water and allowed some time to hydrate before standing for 30 min in a

boiling water bath. It was then homogenized using a food blender and centrifused at 10 000 *g* for 3 min. The pellet was resuspended in 300 ml of hot water and the extraction procedure was repeated until no further polysaccharide was detectable in the supernatant. The extracts were then pooled and precipitated in two volumes of cold acetone. After redissolving in hot water, the polymer solution was ultracentrifuged at 100 000 *g* and 40°C for 90 min and reprecipitated in two volumes of ethanol. The precipitate was collected on sintered glass and washed with ethanol, acetone and ether before freeze-drying.

Chemical analyses

The neutral sugar content of the materials was determined by HPLC using the method of Sutherland and Kennedy (1986). Sugars were separated at 85°C on a Brownlee Polypore PB analytical cartridge (4·6 mm i.d. × 22 cm; Anachem Ltd, Luton, UK). The mobile phase was de-ionized water and the flow rate 0·2 ml/min. Detection of sugars was by means of a Knauer differential refractometer.

The glucuronic acid content of the polymers was determined colorimetrically using the carbazole assay (Bitter & Muir, 1962).

The levels of acetyl and pyruvate substitution were determined (as the free acids) by the hydroxamic acid (Hestrin, 1949) and 2,4-dinitrophenylhydrazine (Sloneker & Orentas, 1962) methods, respectively. All assays were performed at least in triplicate and the results are expressed as a percentage of the total carbohydrate, as determined by the phenol–sulphuric acid assay (Dubois *et al.*, 1956). Assuming only one acyl group per side-chain, the stoichiometric amounts of acetyl and pyruvate are 5·0 and 8·1%, respectively.

Physical analyses

The molecular weight of three native materials was determined by static light scattering. The intrinsic viscosity $[\eta]$ was determined in 20 mM NaCl using a Contraves Low Shear (LS30) couette viscometer. Details of these procedures are given elsewhere (Shatwell *et al.*, 1990*a*).

The helix–coil transition midpoint was determined for a 0·3% xanthan solution in de-ionized water. Measurements were made in a Perkin-Elmer 241 polarimeter, using 10-cm, quartz thermostatted cells, at a wavelength of 365 nm. Further details are given in Shatwell *et al.* (1990*b*).

Estimation of minimum gelling concentrations

Gels comprising 0·5% (w/w) xanthan and 1·0% (w/w) LBG were prepared as follows. The polymers were weighed into a screw-top universal bottle together with the distilled water (total weight, 15·0 g), and the materials were allowed to stand overnight so that the polymers became well hydrated; this facilitated easy dispersion. They were then heated to 80°C in a water bath and sheared vigorously for 5 min at high speed using a top-drive Atomix. Air bubbles were removed by centrifuging for 2 min at 10 000 *g* in a bench centrifuge, and the mixture was reheated to 80°C. Serial 1/2 dilutions were made by dispensing 5·0 ml quantities of the melted gel system into preheated 5·0 ml aliquots of water and homogenizing with a vortex mixer. Dilutions down to 1/64 (i.e. approximately 0·008% xanthan and 0·016% galactomannan) were prepared and allowed to age for 24 h at room temperature before being examined for gel formation.

Mechanical spectrometry

In this technique, a sinusoidal input (strain) wave is applied to a cylindrical sample, and the phase and amplitude of the sinusoidal response (stress) wave are measured. The in-phase (G') and out-of-phase (G'') moduli are determined as a function of strain and frequency. Measurements are made at 'small deformation' so that gel formation can be monitored without perturbing the structure. Other parameters calculated include tan δ, with δ being the phase angle difference, and η^* the dynamic viscosity: for more details see Ross-Murphy (1984).

The response of mixed gel systems to oscillatory shear was measured using the Rheometrics Mechanical Spectrometer RMS-605 with automatic computation and plotting of results. In the present experiments, 50-mm-diameter parallel plates were used (with a gap of 1 mm) in combination with the normal (TC-2000) transducer. Samples comprising 0·5% (w/w) xanthan and 1·0% (w/w) LBG were prepared by dissolving the freeze-dried materials in de-ionized water (total weight, 15·0 g) in screw-top universal bottles. Dissolution was achieved by allowing the polymers to become hydrated overnight, then heating to 85°C in a water bath and shearing for 5 min at high speed using a top-drive Atomix. The samples were heated under pressure for 5 min at 110°C and sheared again for 5 min at maximum speed. Air bubbles were removed by centrifuging at 10 000 *g* for 2 min in a bench centrifuge, and the gel was remelted and loaded on to the instrument. Samples were routinely loaded at 85°C and the gap around the periphery of the sample was

sealed with a light silicon oil (Dow Corning 200/10 cs) to prevent water loss. The temperature was adjusted as detailed below.

RESULTS

Chemical and physical analyses

A range of xanthans with varying levels of acetyl and pyruvic acid substitution were prepared. These materials were analysed using a range of chemical and physical techniques as described above. Table 1 gives a summary of these data.

With the exception of ps.BD9A, all samples had close to the expected sugar ratio for xanthan, i.e. 2 glucose: 2 mannose: 1 glucuronic acid. Sample ps.BD9A contained significantly less mannose than normal xanthan which, combined with its low pyruvic acid content, indicated the probable loss of the terminal mannose residue from the trisaccharide side-chains.

Samples ps.646, ps.1128 and ps.556 had quite similar molecular weights, but evidence from intrinsic viscosity measurements and from analysis of the diffusible material from the reaction mixtures after deacetylation and depyruvylation indicated some reduction in the molecular weight of the native material after chemical modification. The procedure used for depyruvylation, in particular, is believed to have brought about a significant degree of depolymerization.

The LBG sample used contained 19% galactose and 81% mannose, slightly lower than values reported by McCleary *et al.* (1985), but in good agreement with those of Tako *et al.* (1984). $[\eta]$ in 20 mM NaCl was 7·1 dl/g, which corresponded to a molecular weight of $8{\cdot}2 \times 10^5$ estimated using the Mark–Houwink parameters of Robinson *et al.* (1982) for guar, (this gives a molecular weight of 7×10^5 when corrected for the reduced galactose content).

Oscillatory-shear measurements

Oscillatory-shear measurements were carried out on mixed systems comprising 0·5% (w/w) xanthan and 1·0% (w/w) LBG in de-ionized water. The xanthans used were ps.646, (a typical wild-type xanthan) ps.1128, deacetylated (DA) ps.1128 batches 1 and 2, ps.556, depyruvylated (DP) ps.556 and ps.BD9A. These materials represented the complete range in terms of acetyl and pyruvic acid substitution and

TABLE 1
Chemical Composition, Intrinsic Viscosity and Melting Temperature (T_m) of Xanthans

Xanthan	*Glc:Man:GlcA*	*Acetyl substitution (%)*	*Pyruvic acid substitution (%)*	T_m (°C)	*Molecular weight*	$[\eta]$ (dl/g)
ps.646	2·00:1·80:1·10	4·5	4·4	44·0	$0·9–1·2 \times 10^6$	34·1
DA ps.646	2·00:1·96:1·17	1·3	3·6	31·5	—	29·8
DP ps.646	2·00:1·82:1·13	4·4	0·6	51·5	—	15·5
ps.1128	2·00:1·82:1·06	7·7	1·7	54·5	$1·27 \times 10^6$	79·5
DA ps.1128 [a]	2·00:2·10:1·17	1·6	1·3	42·0	—	24·5
DA ps.1128 [b]	2·00:2·07:1·13	1·5	1·3	—	—	22·1
ps.556	2·00:1·91:0·95	1·6	6·0	38·0	$1·48 \times 10^6$	53·8
DP ps.556	2·00:1·65:0·85	1·1	1·0	41·0	—	16·4
ps.BD9A	2·00:0·80:0·77	2·3	2·0	—	—	8·9

[a] Batch 1.
[b] Batch 2.

illustrated the effects of deacetylation and depyruvylation upon specific polymers. Two samples of deacetylated ps.1128 were included in the study to give an indication of the reproducibility of the data. These samples were prepared by deacetylating two separate batches of ps.1128 produced under identical conditions, and the polymers were found to have a very similar chemical composition and intrinsic viscosity.

The following standard set of experiments was performed on each mixed sample of xanthan and LBG. (Identical conditions were used for xanthan–guar and xanthan–konjac mannan mixtures, described in Parts II and III, respectively.)

1. The samples were loaded at 85°C and the temperature was then adjusted to 75°C and held there for 5 min. It was subsequently reduced at a rate of 1°C/min down to 25°C and held there for a further 30 min. G′, G″ and tan δ were monitored throughout the cooling process, using 10% strain and a frequency of 10 rad/s.
2. After 30 min ageing a strain sweep was performed at 25°C. G′, G″ and tan δ were measured over a range of strains between 0–25%, using a frequency of 10 rad/s. This was necessary to ensure that, at the strain used in the other experiments (i.e. 10%), the behaviour of the system was not heavily strain dependent.
3. A frequency sweep was performed next. G′, G″ and η^* were measured over a range of frequencies between 0·01–100 rad/s, using a value of 10% strain.
4. Finally, the sample was reheated to 75°C at a rate of 1°C/min, and G′, G″ and tan δ were monitored using the same deformation conditions as for the cooling sweep.

Figure 1 shows a typical cooling sweep for a mixture of xanthan (in this case ps.646) and LBG. The behaviour is characteristic of a thermoreversible gel system (Clark & Ross-Murphy, 1987). At 75°C (i.e. at the far left of the trace) the gel is in the melted state and the system behaves as a liquid, G″ being significantly greater than G′. As the temperature drops and the gel begins to form, G′ increases sharply and tan δ falls as the system becomes more elastic; G″ also increases, although by less than its elastic counterpart. This phenomenon is usually observed upon gelation. Gel networks typically set up at between 40 and 50°C. G′ continued to increase and tan δ to decrease long after the gel network had become established; in Fig. 1 the two are still changing at the end of the 30-min ageing time. This apparent gradual increase in gel strength over a long period of time has been reported by other authors. Kovacs (1973) noted that for a 1% xanthan–LBG mixture it was 4 h before the increase in gel strength began to level off and, even then, it continued to

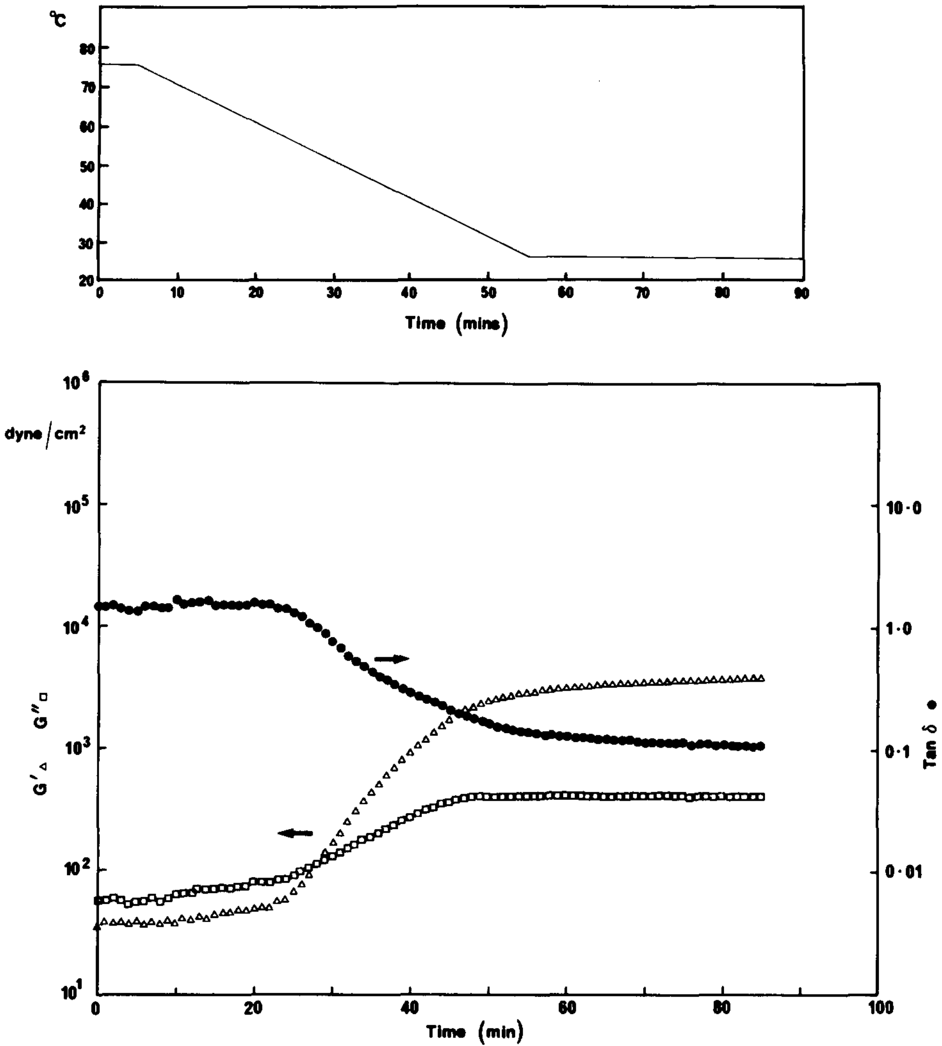

Fig. 1. Temperature sweep and cooling profile, for a mixture of 0·5% ps.646 and 1·0% LBG.

increase very slowly for a further 6–7 days. A plot of tan δ against temperature on cooling and reheating showed significant hysteresis between the two curves; this may be attributed to the increase in gel strength with time.

The frequency sweep for the same system is given in Fig. 2. This is quite clearly that of a strong gel network. G' is much greater than G'', and both show very little frequency dependence. η^* decreases markedly with

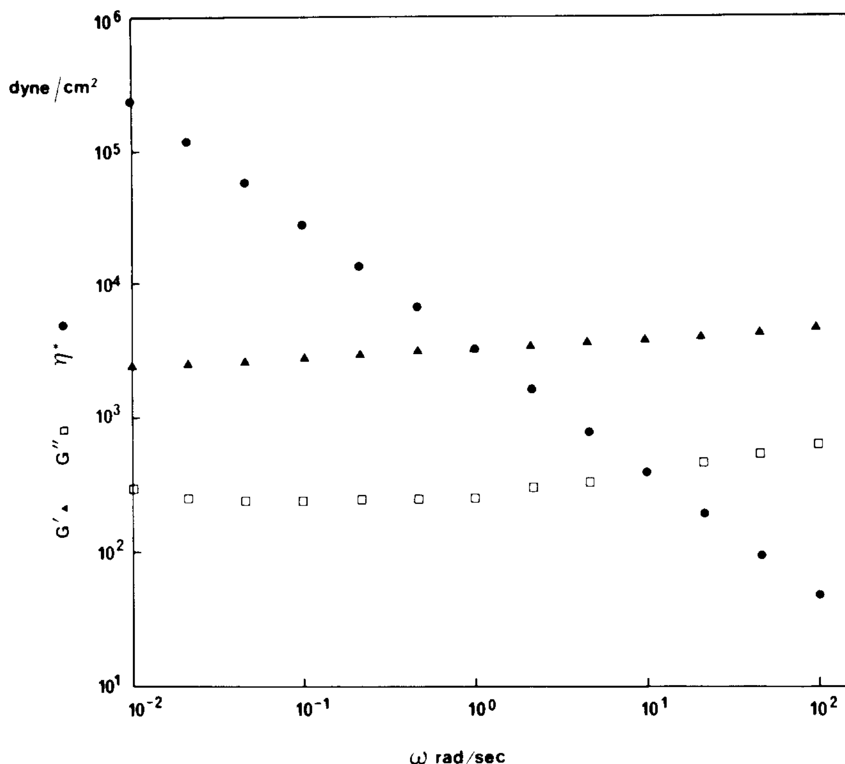

Fig. 2. Frequency sweep for a mixture of 0·5% ps.646 and 1·0% LBG.

increasing frequency (ω). The G′ plot is essentially linear, but that of G″ increases slightly at both the highest and the lowest frequencies. This may be an indication of some sort of relaxation phenomenon, although the precise nature of such a phenomenon at the molecular level, is not understood.

The behaviour of the other xanthans was broadly similar to that of ps.646 with the following exceptions; in the case of ps.1128 the transition from the liquid to the gel state upon cooling was significantly less sharp than that of the other system, and the frequency sweep for ps.BD9A (Fig. 3) indicates a substantially weaker gel network.

Comparative gel strengths

Three measures of gel strength were used in this study — G′ and tan δ at the end of the cooling sweep (i.e. at 25°C, 10% strain and 10 rad/s), and the slope of η^* in the frequency sweep.

Generally speaking, the stronger the gel, the higher the elastic modulus. However, G′ is not an absolute measure of gel strength. It can depend a great deal upon the frequency at which it is measured. A viscous solution, for example, may at appropriate frequencies have a higher G′ value than a true gel network. The latter has associated with it an equilibrium modulus, that is, an elastic modulus at zero ω and zero strain; a Newtonian solution or an entanglement system does not. When comparing different systems one would ideally measure the equilibrium modulus, since this eliminates any problems of frequency or strain dependence, but, of course, this is not possible. Some workers have attempted to determine a pseudo-equilibrium modulus by extrapolation of the G′ plot. In this study, G′ at $\omega = 10$ rad/s was taken as a comparative measure of gel strength. This was satisfactory for the majority of the mixed gel systems where there was little frequency dependence. However, for the weaker systems, in particular ps.BD9A, the dependence of G′ increased at the higher frequencies due to a greater contribution from dynamic entanglements. It should be borne in mind, therefore, that for such systems, G′ at 10 rad/s actually tends to under-emphasize the difference between the weaker and the stronger networks.

The tan δ value at 25°C is a measure of the elasticity of the system. The lower the tan δ value, the more elastic the gel network. Likewise, the greater the slope of log η^* versus log ω, the more elastic the gel. (A

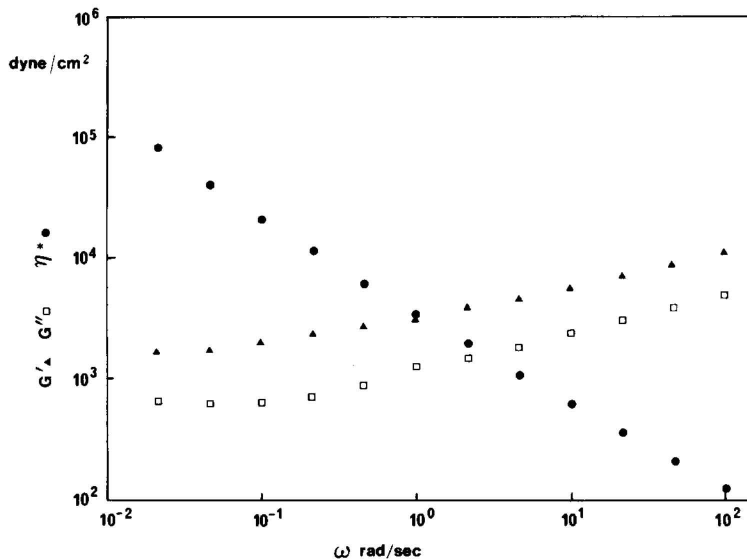

Fig. 3. Frequency sweep for a mixture of 0·5% ps.BD9A and 1·0% LBG.

perfectly elastic system has a slope of -1, because $\eta^* = G^*/\omega$, and for such an elastic system G^* is independent of ω.)

Table 2 gives a summary of the data for each system. The extremely good agreement between the data for the samples of deacetylated (DA) ps.1128 suggests that the overall reproducibility of the data was good.

The majority of the xanthans tested formed a relatively strong gel network with LBG, but sample ps.1128 was an exception. G' and the slope of η^* were significantly lower than for most of the other systems, and tan δ was somewhat higher. Deacetylation of ps.1128 resulted in a significant increase in gel strength. G' increased by roughly 250%, tan δ fell, and the slope of η^* increased from $-0{\cdot}88$ to $-0{\cdot}95$. In contrast, depyruvylation of ps.556 hardly produced any change. This suggests that the acetyl group has an inhibitory effect upon gelation, but that the pyruvate group plays no role in the gelling interaction.

The polymer from the mutant strain BD9A produced a much weaker gel than any of the other xanthans tested. G' for this system was exceptionally low, tan δ very high and the slope of η^* low. In addition, as already noted, this polymer showed increasing frequency dependence at the higher ω values. Both observations suggest that the terminal mannose residue on the trisaccharide side-chain plays an important role in the xanthan–LBG interaction.

Table 3 shows the results of the minimum gelling concentration experiments. Serial 1/2 dilutions were performed on the xanthan–LBG systems in the melted state. After cooling they were assessed visually for gel formation and placed into one of the following four categories: strong gel (+ +), maintained its shape unsupported and was firm to the touch;

TABLE 2

Oscillatory-Shear Measurements for Xanthan (0·5%)–LBG (1·0%) Mixed Systems

Xanthan	*tan δ*	*G′*	*Slope of η**
ps.646	0·095	4300	−0·92
ps.1128	0·25	930	−0·88
DA ps.1128 [a]	0·049	3300	−0·95
DA ps.1128 [b]	0·049	3800	−0·94
ps.556	0·048	5100	−0·94
DP ps.556	0·049	5200	−0·93
ps.BD9A	0·41	620	−0·88 (LF) −0·69 (HF)

LF — low frequencies, HF — high frequencies.
[a]Batch 1.
[b]Batch 2.

TABLE 3
Estimated Minimum Gelling Concentrations for Xanthan–LBG Mixed Systems

		Gelation						
	Dilution	*0*	*1/2*	*1/4*	*1/8*	*1/16*	*1/32*	*1/64*
	~ % LBG	*1·0*	*0·5*	*0·25*	*0·125*	*0·063*	*0·031*	*0·016*
Xanthan	*~ % Xan*	*0·5*	*0·25*	*0·12*	*0·063*	*0·031*	*0·016*	*0·008*
ps.646		+ +	+ +	+	+	+/−	−	−
DA ps.646		+ +	+ +	+	+	+/−	−	−
DP ps.646		+ +	+	+	+/−	−	−	−
ps.1128		+	+	+/−	−	−	−	−
DA ps.1128[a]		+ +	+ +	+	+	+	+/−	−
ps.556		+ +	+ +	+ +	+	+	+/−	−
DP ps.556		+ +	+ +	+	+	+/−	−	−

[a]Batch 1.

soft gel (+), incapable of supporting its own weight against gravity and yielded readily to compression; structured solution (+ / −); free-flowing solution (−).

Deacetylation of ps.1128 caused a marked decrease in the minimum gelling concentration of the polymer with LBG. The native xanthan gelled with the galactomannan down to a minimum total polysaccharide concentration of 0·3% but the deacetylated derivative gelled at a total concentration as low as 0·094%. Sample ps.556, a native low-acetyl, high-pyruvate polymer, also gelled down to a minimum total concentration of 0·094%. Deacetylation of ps.646 produced no apparent change in minimum gelling concentration, but the gels formed by the deacetylated derivative were found to be firmer to the touch than those of the native polymer at equivalent concentrations.

Depyruvylation of both ps.556 and ps.646 brought about a slight increase in the minimum gelling concentration, suggesting that depyruvylation causes a slight decrease in gel strength.

Statistical analysis

Virtually all of the data presented so far indicate that the strength of the interaction between xanthan and LBG is heavily dependent upon the level of acetyl substitution. The role of pyruvate, however, is much less clear cut. An attempt was therefore made to look for statistically significant correlations between the gel strength and the levels of acetyl and pyruvate substitution. The molecular weight, as indicated by $[\eta]$, was also considered.

Using PROC RSQUARE from the SAS library (SAS, 1985), a multiple-regression analysis was carried out on the oscillatory-shear data omitting that for ps.BD9A. The model-dependent parameters, i.e. tan δ, G' and slope of η^*, were regressed against the independent variables, acetyl, pyruvate and $[\eta]$, and the full correlation matrix of dependent and independent variables was computed (Table 4). For the purpose of this study, values of 0·85 and above, or $-0{\cdot}85$ and below, may be considered a reasonable indication of statistical significance.

Significant correlations were found between tan δ and G', and tan δ and the slope of η^*, these all being measures of 'gel strength'. Correlations were also found between the percentage acetyl substitution and both tan δ and the slope of η^*. This supports the view that acetyl has a significant influence upon the strength of the interaction with LBG. No correlations were found between gel strength and either the percentage pyruvate or $[\eta]$. However, there was evidence to suggest that certain of the $[\eta]$ values may have been influenced by the presence of high-molecular-weight aggregates formed on freeze-drying. This was undoubtedly true in the case of ps.1128 (Shatwell, 1989) and, for this reason, the statistical data relating to $[\eta]$ may not be completely reliable.

An attempt was made to investigate whether or not there was a statistically significant correlation between the gel strength and the helix–coil transition midpoint (T_m). Using PROC RSQUARE, the tan δ, G' and slope of η^* values for the mixed systems were regressed against the helix–coil transition midpoints given in Table 1. The resultant correlation matrix is shown in Table 5. A strong positive correlation was found between T_m and both tan δ and the slope of η^*. A strong negative correlation was obtained between T_m and the G' value, i.e. the higher the transition midpoint, the weaker the gel formed with LBG. Whether or not these two observations are directly related cannot be established at this time. (T_m for xanthan depends upon the level of acetyl and pyruvate substituents, and the concentration of associated counterions.)

Whilst the statistical approach we have applied is novel to this area, in some respects the results finally obtained were disappointing. The most obvious reason for this was that a relatively small number of xanthan samples were employed (six), and the variations in composition were also (naturally) rather limited. Nevertheless, some new insight was gained, and further remarks are left to the discussion.

DISCUSSION

In line with the findings of Dea *et al.* (1977) and Tako *et al.* (1984) we have demonstrated that the acetyl substituent has a marked inhibitory

TABLE 4

Correlation Matrix Relating Acetyl, Pyruvate and Intrinsic Viscosity to Gel Strength in xanthan (0·5%)–LBG (1·0%) Mixed Systems

	Acetyl substitution (%)	*Pyruvic acid substitution (%)*	*[η]*	*tan δ*	*G′*	*Slope of η**
% Acetyl substitution	1·000	0·019	0·799	0·965	−0·804	0·939
% Pyruvic acid substitution		1·000	0·329	−0·126	0·387	−0·086
[η]			1·000	0·832	−0·655	−0·764
tan δ				1·000	−0·865	0·962
G′					1·000	−0·716
Slope of η*						1·000

TABLE 5
Correlation Matrix Relating Gel Strength to the Helix–Coil Transition Midpoint

	tan δ	*G′*	*Slope of η**	T_m
tan δ	1·000	−0·891	0·960	0·973
G′		1·000	−0·739	−0·927
Slope of η*			1·000	0·921
T_m				1·000

effect upon the interaction of xanthan with LBG at low ionic strength. Such inhibition of intermolecular association by acyl groups is quite common in biopolymer systems (Morris & Miles, 1986). When salt is added to an aqueous solution of XM-6, an exocellular polysaccharide produced by *Enterobacter* (NCIB 11870), a thermoreversible gel is formed (Nisbet *et al.*, 1984). However, the capsular polysaccharide from *Klebsiella aerogenes* serotype K54, a naturally acetylated polymer with the same basic tetrasaccharide repeat unit as XM-6, will not form a gel unless it is first chemically deacetylated (O'Neill *et al.*, 1986). Gellan gum, a glyceryl substituted polysaccharide secreted by *Pseudomonas elodea*, will gel both in the native state and after deacetylation, but the texture of the gel is significantly different (Moorhouse *et al.*, 1981). The native polymer forms a soft, elastic gel after heating and cooling, whereas that of the deacetylated derivative is firm, non-elastic and rather brittle. The difference in texture is believed to be due to a much stronger interaction between the deacetylated molecules. Native konjac mannan will not gel alone, but will do so when warmed in the presence of alkali. Gelation is believed to be due to the elimination of the acetyl groups under alkaline conditions (Maekaji, 1974). Acetyl groups have also been shown to inhibit gelation in plant pectin (Pippen *et al.*, 1950).

The reason for inhibition in these other systems is not well understood. For konjac mannan (Cairns *et al.*, 1988), the K54 capsular polysaccharide (Atkins *et al.*, 1987) and gellan gum (Carroll *et al.*, 1982), X-ray fibre-diffraction studies have shown that the ordered conformation of the polymers is not significantly affected by deacetylation and that the only change is an increase in the propensity of the polymers to co-crystallize. Exactly how the acetyl group inhibits co-crystallization has still to be established. In the case of konjac mannan, Maekaji (1974) has suggested that the acetyl group suppresses intermolecular hydrogen bonding and that its removal decreases the solubility of the polymer by enhancing intermolecular association, leading eventually to gelation.

Thus, despite the frequency with which acetyl is found to inhibit gelation, examination of other biopolymer systems throws very little light upon the role of the acetyl group in xanthan–LBG gels. The absence of a well-established mechanism for the interaction makes speculation more difficult. There are three ways in which the acetyl group could inhibit intermolecular association. The first is direct steric hindrance. The second is suppression of the intermolecular attractive forces, particularly hydrogen bonding, as has been suggested for konjac mannan. (The weakening of the gelling interaction between xanthan and LBG in the presence of urea (Tako *et al.*, 1984; Cheetham & Mashimba, 1988), suggests that hydrogen bonding may play an important role in the interaction). The third possibility is that the acetyl group influences the gelling properties of xanthan through its effect upon the ordered conformation. Acetyl has been shown to stabilize the xanthan helix and to raise the helix–coil transition midpoint (Dentini *et al.*, 1984; Shatwell *et al.*, 1990*b*). There is also limited evidence to suggest that high acetyl polymers are less flexible than low acetyl materials in solution (Coviello *et al.*, 1986). Tako *et al.* (1984) have suggested that an increase in the flexibility of the xanthan molecule upon deacetylation could facilitate easier association between the xanthan side-chains and the galactomannan backbone. The model of Cairns *et al.* (1986, 1987) states that gelation can only occur when xanthan is in the disordered state, and this implies that by raising the helix–coil transition midpoint the acetyl group could limit the ability of the polymer to gel under certain conditions. The strong negative correlation observed between T_m and gel strength, for the xanthan–LBG systems, would be consistent with this view.

However, the evidence presented in this study does not favour any one of the three current models preferentially. Indeed, given the seemingly contradictory nature of much of the available experimental evidence, none of the present models seems entirely satisfactory.

The role of pyruvic acid in the xanthan–LBG interaction is not conclusive. A slight increase in the minimum gelling concentration of the polymers after depyruvylation suggested that pyruvate may promote gelation, but the oscillatory-shear measurements on ps.556 and its chemically modified derivative, together with the statistical analysis of the data, do not support this view. Consideration of the data is further complicated by the concomitant decrease in molecular weight which is believed to have occurred upon depyruvylation, and by the possibility that some of the pyruvic acid was lost from the polymer when the sample was autoclaved (Cheetham & Punruckvong, 1985). The latter, although it could theoretically have had a significant effect on the results, is unlikely to have done so, since oscillatory-shear measurements carried out on

unautoclaved samples of ps.646 and LBG differed no more from those of the autoclaved samples than by normal sample-to-sample variation.

The effect of molecular weight on gel strength has so far been largely ignored. No obvious correlation was found between the intrinsic viscosity of xanthan and the gel strength, and this was confirmed by the statistical analysis. However, the suitability of intrinsic viscosity as a measure of molecular weight has been called into question. In theory, the rigidity of a polymer gel should increase with the molecular weight, since the chance of macromolecular association rises as the length of the molecules increases (Janus & Flory, 1974). In practice though, this appears only to be true in the low-molecular-weight region. Data presented by Smidsrød (1974) for alginate gels, namely a plot of modulus of rigidity against degree of polymerization, showed that for this system, above a minimum critical degree of polymerization (approximately 500), the modulus of rigidity did not change. This is consistent with the view that above a minimum critical molecular weight the concentration of cross-linkages remains fixed as the primary molecular weight increases (Janus & Flory, 1974). What the minimum critical molecular weight for xanthan would be in xanthan–LBG gels is unknown. There is, therefore, no way of telling whether the apparent reduction in the molecular weight of xanthan after depyruvylation could have been responsible for any decrease in gel strength. The problem does not arise in the case of deacetylation since here the gel strength appeared to increase in spite of possible depolymerization. Interestingly, marked differences in gel strength were recorded for the systems prepared with ps.646, ps.1128 and ps.556, and yet the molecular weights of these polymers, determined by light scattering, were quite similar. This confirms that over the molecular weight range $0{\cdot}9–1{\cdot}48 \times 10^6$, at least, factors other than the molecular weight were of overriding importance.

Finally, of all the xanthan–LBG interactions studied, that of ps.BD9A was by far the weakest. Sample ps.BD9A is believed to lack the terminal mannose residue from the trisaccharide side-chain, suggesting some fundamental role for the side-chain in the xanthan–galactomannan interaction. The side-chain could be directly involved in binding, as Tako *et al.* (1984) have suggested; alternatively, assuming that the ordered helical conformation is necessary for xanthan–galactomannan binding (Dea *et al.*, 1977), loss of the terminal mannose residue could have inhibited the interaction by preventing adoption of the ordered state. The helix–coil (OR) transition curve for ps.BD9A indicated that, in deionized water, the polymer was capable of adopting at least a partially ordered conformation. However, at 25°C, the temperature at which the

oscillatory-shear measurements were made, the polymer had already lost a substantial amount of order. The very low intrinsic viscosity of ps.BD9A i.e. 8·9 dl/g in 20 mM NaCl, suggests that the molecular weight of this polymer was also quite low, which might provide an alternative explanation for the very weak interaction.

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