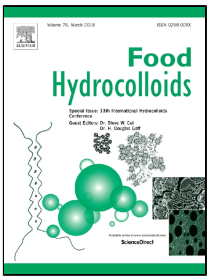
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Ordered conformation of xanthan in solutions and "weak gels": single helix, double helix – or both?

Edwin R. Morris

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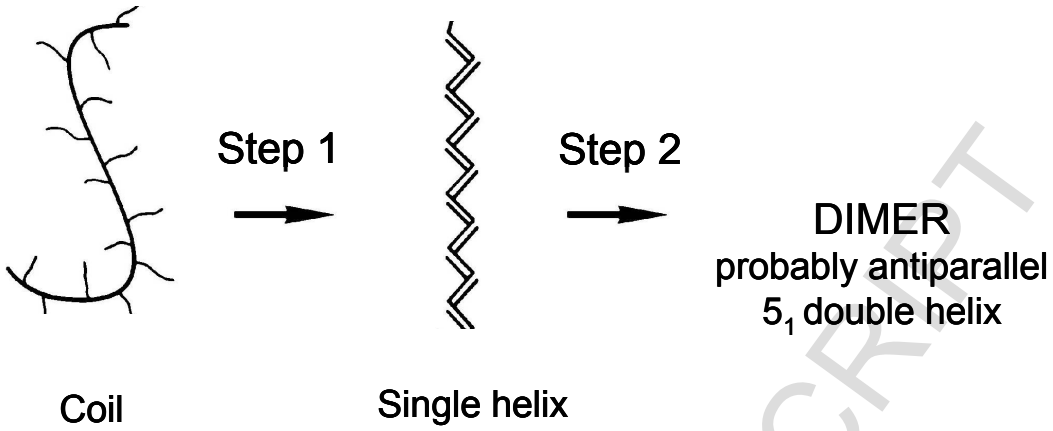
Highlights

- Rapid progress of xanthan from recent discovery to major food hydrocolloid
- Accompanying evidence of both single-helix and double-helix structures
- Proposed interpretation in terms of 2-stage process of conformational ordering
- Stage 1: coil–single helix transition; stage 2: conversion to 5_1 double helices

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Graphical Abstract



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Ordered conformation of xanthan in solutions and "weak gels": single helix, double helix – or both?

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Abstract

During the progress of xanthan from a recent discovery to its present status as a well-established food hydrocolloid, some investigations have indicated that the ordered structure, which underlies most of the practical applications of xanthan, is a single helix stabilised by ordered packing of sidechains along the polymer backbone, giving first-order kinetics for the disorder–order transition. Others favoured a coaxial double helix, whose formation causes a doubling of molecular weight and mass per unit length. It is proposed here that both interpretations are correct, and that ordering is a 2-stage process: formation of single helices followed, under favourable conditions, by enthalpically-driven conversion to coaxial double helices. Comparison of recent evidence from atomic force microscopy with models from analysis of X-ray fibre diffraction data suggests that the most likely coaxial arrangement is an antiparallel 5_1 double helix.

Keywords

Xanthan; Conformation; Single helix; Double helix; Light scattering; Optical rotation

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1. Introduction

Although now firmly established as one of the repertoire of polysaccharides available for use in food, xanthan is a comparatively new arrival. Indeed, its progress from a recent discovery to one of the major food hydrocolloids occurred during the span of my own scientific career.

As background for addressing the question raised in the title, this article gives a historic account of the accompanying progress in developing molecular understanding of the functional properties of xanthan, including my own personal involvement in the xanthan saga. It is based closely on a lecture I gave at the international conference "Gums and stabilisers for the food industry 19", Berlin, 27-30 June, 2017.

2. Discovery of xanthan

Xanthan was identified as a potentially useful material during an extensive bacterial screening programme carried out in the USDA Northern Regional Research Laboratory in Peoria, Illinois (Jeanes, Pittsley & Senti, 1961). The work had two complementary aims: (i) to reduce the reliance of the USA on imported gums; and (ii) to develop a new outlet for US corn production by using hydrolysed corn (maize) starch as growth substrate for the bacteria.

The screening programme was led by Dr. Allene Jeanes, and also resulted in the discovery of dextran, used as a plasma-extender in blood transfusions, notably for treatment of American troops wounded in the Korean conflict. For her services to US science, Dr. Jeanes received the Federal Women's Service Award in 1962 (Fig. 1a).

Xanthan is an anionic exopolysaccharide produced in high yield by the bacterium *Xanthomonas campestris* (cabbage blight) on aerobic fermentation. In early publications it was known as polysaccharide B-1459, and was first produced commercially by Kelco, San Diego (now part of CP Kelco) as Kelzan (for industrial use, mainly in the oil industry) or Keltrol (food grade). Since expiry of Kelco's patent protection it has become commercially available from other suppliers, notably in China.

3. Start of my own involvement with xanthan

In 1970, after completing my PhD on an entirely different topic (gas-phase reaction kinetics) in Edinburgh University, I was taken on for a Post-doc with a charismatic young lecturer, Dr. D.A. Rees (Fig. 1b), now Sir Dai Rees, FRS, who was carrying out ground-breaking research on polysaccharide conformation. This included showing that the intermolecular junctions in iota carrageenan gels are double helices whose formation and melting are

accompanied by large, sigmoidal changes in optical rotation (McKinnon, Rees & Williamson, 1969; Rees, 1972).

A few months after I had started my project, Dr. Rees was head-hunted by Unilever, and asked me to join his new group in their Colworth Laboratory in Bedfordshire, UK. Early in 1971 I was invited to visit Dr. Allene Jeanes in Peoria to hear about her work on xanthan, and shortly after received a sample from one of the first production batches of Keltrol.

In view of the success of optical rotation in detecting changes in conformation of carrageenan, I tried the same approach for xanthan. To economise on polarimeter time, I built a rather cumbersome, but effective, device to run the instrument overnight, with automated control of temperature and direct output of point-by-point measurements on an X–Y recorder (this being long before laboratory computers were available to control instruments and record data). One of the recorder traces obtained (complete with the original hand-lettering) for a solution of xanthan on repeated cycles of heating and cooling is reproduced in Fig. 2a, and shows a thermally-reversible sigmoidal transition with no detectable thermal hysteresis. This was the first direct evidence that xanthan undergoes a conformational transition on heating and cooling, and I reported it briefly in a book chapter (Morris, 1973), along with preliminary accounts of ongoing research in Colworth on the conformation and functional interactions of other polysaccharides.

The changes in optical rotation shown in Fig. 2a are accompanied (Fig. 2b) by loss of detectable high-resolution NMR signal on cooling (Morris, Rees, Young, Walkinshaw & Darke, 1977), demonstrating conversion from mobile, disordered coils at high temperature to a rigid, ordered conformation. The disorder–order and order–disorder transitions of xanthan have been characterised subsequently (e.g. Holzwarth, 1976; Milas & Rinaudo, 1979; Morris et al., 1977; Muller, Anrhourrache, Lecourtier & Chauveteau, 1986; Norton, Goodall, Frangou, Morris & Rees, 1984) by a wide range of physical techniques, including circular dichroism (CD), differential scanning calorimetry (DSC), potentiometric titration, viscosity measurements, and light scattering, as well as optical rotation and NMR.

In addition to thermal denaturation, ordered xanthan can be converted to the disordered coil form by cadoxen (Sato, Norisuye & Fujita, 1984), a strongly alkaline solvent which is capable of dissolving cellulose.

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4. Rheology of ordered xanthan

Solutions of xanthan have unusual, characteristic, rheological properties (Whitcomb & Macosko, 1978) which can be traced to weak association of the ordered structures. Figure 3a shows mechanical spectra from low-amplitude oscillation for typical polysaccharide solutions at concentrations where the individual coils are forced to interpenetrate and entangle with one another (as in most practical applications), and conventional gel networks. For gels, solid-like response, characterised by storage modulus (G'), predominates over viscous flow (loss modulus, G"), both moduli show only slight variation with frequency of oscillation (ω), and complex dynamic viscosity [$\eta^* = (G'^2 + G''^2)^{½} / \omega$] decreases steeply with increasing frequency, with a slope close to -1 on a log–log plot.

For solutions of entangled coils, both moduli increase steeply with increasing frequency, with G' rising above G". A characteristic feature of such solutions is that the frequency-dependence of η^* is closely superimposable on the shear-rate dependence of viscosity (η) from rotational measurements at equivalent numerical values of shear rate (s^{-1}) and frequency (rad s^{-1}). This generality of behaviour is known as "Cox–Merz superposition" (Cox & Merz, 1958).

As shown in Fig. 3b, mechanical spectra of ordered xanthan (Ross-Murphy, 1984; Ross-Murphy, Morris & Morris, 1983) more closely resemble those of gels than of conventional polysaccharide solutions, with G' > G" and little frequency-dependence of either modulus. This combination of gel-like response to small deformation coupled with the ability to flow freely in response to higher stress is known as "weak-gel" rheology (Ross-Murphy, 1984) and underlies most of the practical applications of xanthan (e.g. in pourable dressings that cling to the surface of salad, rather than running off). The "weak gel" rheology of xanthan can be weakened or eliminated by urea, but promoted selectively by different cations (in the order $Na^+ < K^+ < Ca^{2+}$), indicating specific association between the ordered structures to form a continuous network (Ross-Murphy et al., 1983).

A characteristic feature of such "weak gels" is that they violate the Cox–Merz rule: as shown in Fig. 3b, η^* of the gel-like network exceeds η from rotational measurements where the network is broken down by the imposed shear.

5. Primary structure of xanthan

When the disorder–order transition of xanthan was first detected (Fig. 2a), the primary structure of the polymer had not been determined, so the nature of the ordered conformation was unknown. The structure was elucidated by Jansson, Kenne & Lindberg (1975), with

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confirmatory evidence from concurrent research in Unilever (Melton, Mindt, Rees & Sanderson, 1976). Xanthan consists of a linear backbone of (1 → 4)-linked β-D-glucose residues (identical to cellulose), but is solubilised by charged trisaccharide sidechains attached at O(3) of alternate glucose residues to give a pentasaccharide repeating unit (Fig. 4). The sidechains have the structure: β-D-Man*p* - (1 → 4) - β-D-GlcA*p* - (1 → 2) - α-D-Man*p*-(1→, with variable, non-stoichiometric substitution by pyruvate and acetate groups. Pyruvate is attached predominantly to the outer mannose as 4,6-linked ketal substituents and acetate predominantly at C(6) of the inner mannose (as in Fig. 4), but other patterns of substitution have been identified recently (Kool, Gruppen, Sworn & Schols, 2013) and influence the stability of the ordered conformation (Kool, Gruppen, Sworn & Schols, 2014).

6. X-ray fibre diffraction in comparison with early solution studies

Diffraction of X-rays from oriented fibres in the solid state is the only technique that can characterise the ordered structures of polysaccharides at atomic resolution. This approach was applied to xanthan by a group established and led by Professor Struther Arnott (Fig. 1c) in Purdue University, Indiana, USA (Moorhouse, Walkinshaw & Arnott, 1977). Oriented fibres were produced by gradual stretching to induce alignment while drying under conditions of controlled humidity. The diffraction patterns obtained from these fibres showed a 5-fold helix structure (i.e. pentagonal in cross-section) with a pitch of 4.7 nm. The nature of the helix was then explored by computer modelling (Arnott & Mitra, 1984) using these parameters in conjunction with known constraints of ring geometry and required separation between atoms. Best agreement with measured diffraction intensities was obtained for a single-helix structure stabilised by ordered packing of sidechains along the polymer backbone (Fig. 5a).

Adoption of single-helix geometry in solution was proposed by Morris et al. (1977), based on the observation that the temperature of the conformational transition is independent of xanthan concentration. Subsequently, Milas & Rinaudo (1979) found that the hydrodynamic volume of both the ordered and disordered conformations was almost constant on varying temperature, and concluded that "the mechanism proposed by Morris is confirmed, and a multichain process is excluded".

There was, however, conflicting evidence from concurrent investigations. Holzwarth & Prestridge (1977) reported that electron micrographs of disordered xanthan showed strands of width ~2 nm, but the ordered conformation had a width of ~4 nm, indicating a multi-stranded structure, most probably a double helix. Analysis of intrinsic viscosity data from the same

group (Holzwarth, 1978) suggested that the mass per unit length of ordered xanthan is about twice that of the single helix proposed from X-ray.

This evidence of double-helix geometry prompted re-analysis of existing data by the X-ray fibre diffraction group in Purdue (Okuyama, Arnott, Moorehouse, Walkinshaw, Atkins & Wolf-Ullish, 1980). The findings were (i) that contrary to conclusions from the initial analysis by Moorehouse et al. (1977), multistranded structures are sterically feasible, and (ii) that the most likely of these is a 5_1 double helix with the two strands running antiparallel to one another (Fig. 5b). As in the original single-helix model, the polymer chains are highly extended (i.e. not wound together in a tight "spiral staircase" arrangement).

Agreement with observed diffraction intensities was similar for both models. Thus X-ray fibre diffraction does not give conclusive evidence of either single or double helix geometry.

7. Kinetics of the xanthan disorder–order transition

As shown if Fig. 6, the conformational transition of xanthan (like those of other charged polysaccharides) moves to higher temperature with increasing ionic strength. Thus ordering can be induced at fixed temperature by addition of salt. This was the basis of an investigation of the kinetics of the disorder–order transition in a research collaboration between York University, UK and Colworth (Norton et al., 1984). Measurements were made at a temperature where xanthan is disordered in the absence of added salt. The solution of disordered xanthan was loaded into a syringe on custom-built apparatus; a second syringe was filled with a concentrated salt (KCl) solution; both syringes were fired simultaneously through an ultra-high-speed mixer into an observation cell and the time-course of conformational ordering in response to salt was monitored by optical rotation.

Reaction-progress curves (Fig. 7a) were analysed by first-order and second-order kinetic schemes. As shown in Fig. 7b, the first-order plot gave good linearity, arguing for intramolecular conversion from disorder to order (i.e. coil → single helix), whereas the second-order plot showed obvious curvature, which is inconsistent with an intermolecular 2 coil → double helix process.

Linear second-order plots were, however, obtained in the same laboratory, using the same apparatus and same experimental procedure, for iota carrageenan (Norton, Goodall, Morris & Rees, 1983a), kappa carrageenan (Norton, Goodall, Morris & Rees, 1983b) and agarose sulfate (Norton, Goodall, Austen, Morris & Rees, 1986), all of which are known to form

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175 double helices. The first-order kinetics observed for xanthan (Fig. 7) therefore strongly
176 suggests single-helix formation as the rate-limiting process in conformational ordering.

177 **8. Proposed solution to an acrimonious controversy**

178 Later evidence remained inconclusive. For example, Muller et al. (1986), using light
179 scattering, observed no change in molecular weight on conformational ordering of xanthan.
180 However, Sato et al (1984), also using light scattering, found that the molecular weight of
181 ordered xanthan (in 0.1 M NaCl) was about twice that of the disordered form in cadoxen, and
182 analysis of intrinsic viscosity data by the same group (Sato, Kojima, Norisuye & Fujita, 1984)
183 gave a value of mass per unit length for ordered xanthan that agreed well with the value
184 calculated for the 5_1 double helix from X-ray (Fig. 5b).

185 The nature of the ordered conformation of xanthan has been the subject of heated controversy,
186 including the comment "No clear thinking person can consider the ordered xanthan
187 conformation as anything other than a double helix [Professor E.D.T. Atkins, 7th Harden
188 Discussion Meeting, 1992, as quoted by Professor John Mitchell in a report on the conference
189 for Carbohydrate Polymers (Mitchell, 1993)]. There is, however, evidence that the proposals
190 of single-stranded and double-stranded ordered structures may both be correct.

191 Figure 8a shows a direct comparison of the temperature-course of changes in molecular
192 weight from low-angle laser light scattering and the conformational transition monitored by
193 optical rotation (Norton et al., 1984) for the same solution of xanthan (1 mg/ml in 10 mM
194 KCl). Increase (approximate doubling) in molecular weight on cooling occurs only in the final
195 stages of conformational ordering. Similarly, when conformation is varied by varying
196 concentration of cadoxen, change in molecular weight occurs (Fig. 8b) at substantially lower
197 cadoxen concentration than change in optical activity. The two plots in Fig. 8b are taken
198 from Kitagawa, Sato, Norisuye & Fujita (1985), where they are presented in separate frames
199 and the increase in molecular weight (again an approximate doubling) with decreasing
200 concentration of cadoxen was adduced as evidence for conversion from a disordered
201 conformation to a double-helix structure.

202 However, ordering of xanthan cannot occur as a simple 2 coil → double helix process,
203 otherwise change in conformation and increase in molecular weight would follow the same
204 temperature course and show the same dependence on cadoxen concentration. The obvious
205 interpretation is that ordering is a 2-stage process (Fig. 9). The first step is conversion of coils
206 to single helices, an intramolecular process giving rise to the observed first-order kinetics

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(Section 7). The second step is conversion of single helices to dimers, causing doubling of molecular weight.

The dimerisation process envisaged originally (Norton et al., 1984) was side-by-side association into structures analogous to the highly-stable "egg-box" dimers in calcium-induced gels of alginate (Morris, Rees, Thom & Boyd, 1978) and low-methoxy pectin (Morris, Powell, Gidley & Rees, 1982). However, I now suggest that coaxial dimerisation would be more favourable enthalpically, by allowing more-extensive non-covalent bonding to occur between the two participating strands, and is indeed more consistent with recent evidence from atomic force microscopy (Moffat, Morris, Al-Assaf & Gunning, 2016).

Figure 10a shows one of the AFM images obtained. The dominant feature is the presence of long, extended structures with obvious periodicity. Running the probe along the contour length of these structures gave a repeat distance of 4.7 nm, as found by X-ray fibre diffraction (Section 6). There are two clear indications that the structures are dimeric. The feature circled in Fig. 10a shows unravelling of a short stretch of ordered structure into two strands, and separation into two disordered chains at the end of an ordered sequence can be seen at the top right-hand corner of the image. Scanning backwards and forwards along the zig-zag path marked on the micrograph showed the expected large reduction in height on going from the ordered to the disordered regions (Fig. 10b).

The visual impression of a coaxial double-helix structure in Fig. 10a was reinforced by quantitative agreement between the height measured by AFM and the lateral dimensions of the double helix modelled by Okuyama et al. (1980). Other images obtained by Moffat et al. (2016) showed "hairpin loops" at the ends of ordered regions, which is consistent with the antiparallel double-helix structure that emerged as the most likely coaxial arrangement from re-analysis of X-ray fibre diffraction data

The controversy surrounding the ordered structure of xanthan appears to be predicated on the implicit assumption that biopolymers can have only one type of ordered conformation, which is demonstrably wrong. Interconversion of protein sequences between α-helix, β-sheet and non-repeating structures is common, and extensively documented. In the polysaccharide field, the 2-fold "egg box" junctions in calcium pectinate gels convert on drying to a 3-fold conformation (Alagna, Prosperi, Tomlinson & Rizzo, 1986; Morris et al., 1982). The experimental findings summarised here suggest similar interconversion between single-helix and double-helix structures of xanthan.

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9. Conclusions

The main conclusions from this brief review of xanthan conformation can be summarised as follows.

Conformational ordering of xanthan is a 2-step process.

Step 1: conversion of disordered coils to single helices stabilised by ordered packing of side-chains along the polymer backbone. Since this is an intramolecular process, it has first-order kinetics.

Step 2: dimerisation, giving a doubling of molecular weight and mass per unit length. The dimer is most probably the 5_1 antiparallel double helix proposed from re-analysis of X-ray fibre diffraction data.

Under some experimental conditions, the ordering process stops after Step 1, which would explain why some groups have observed a disorder–order transition without increase in molecular weight.

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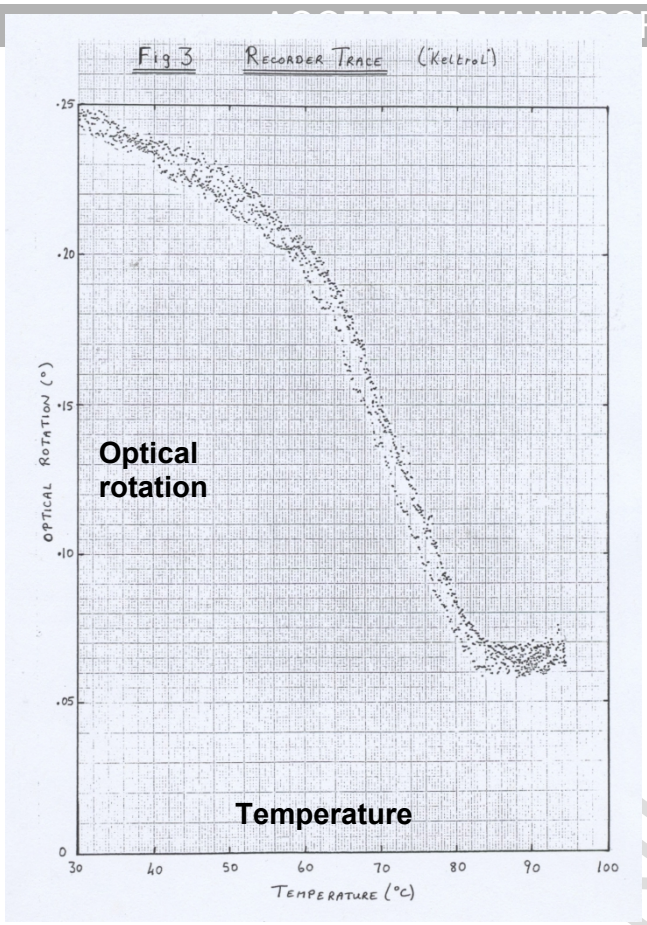


Fig. 1. (a) Dr. Allene Jeanes (circled) receiving the 1962 Federal Women's Service Award from US President John F. Kennedy. (b) Dr. D.A. Rees (ca. 1970). (c) Professor Struther Arnott.

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a



b

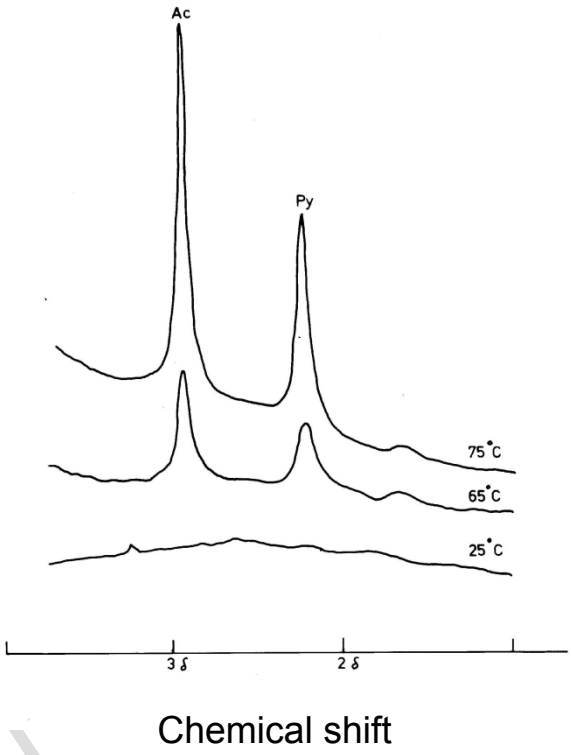
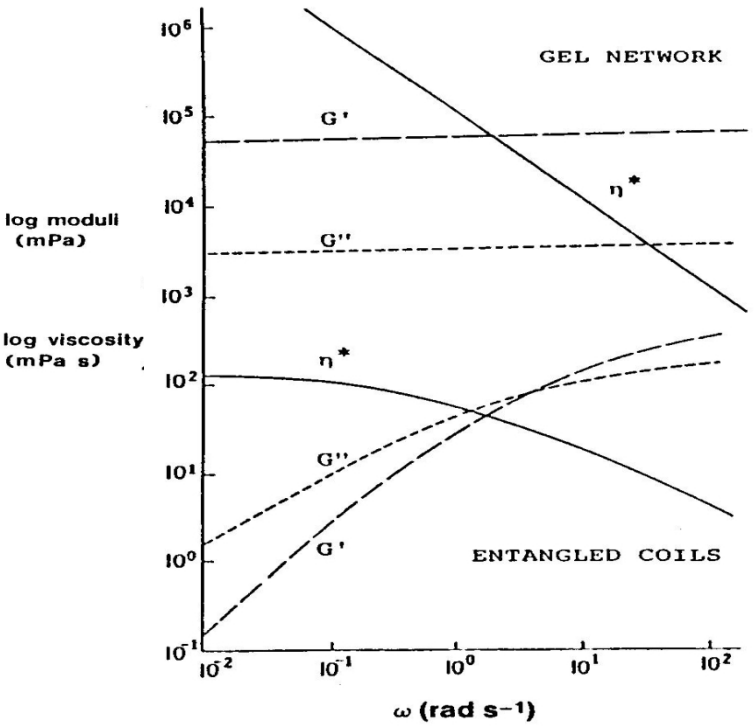


Fig. 2. (a) Recorder trace (including the original hand-lettering for an internal Unilever report), showing the variation of optical rotation with temperature for a solution of xanthan on repeated cycles of heating and cooling (E.R. Morris, 1971, unpublished). (b) Accompanying loss of high-resolution NMR signal on cooling (Morris et al., 1977).

a



b

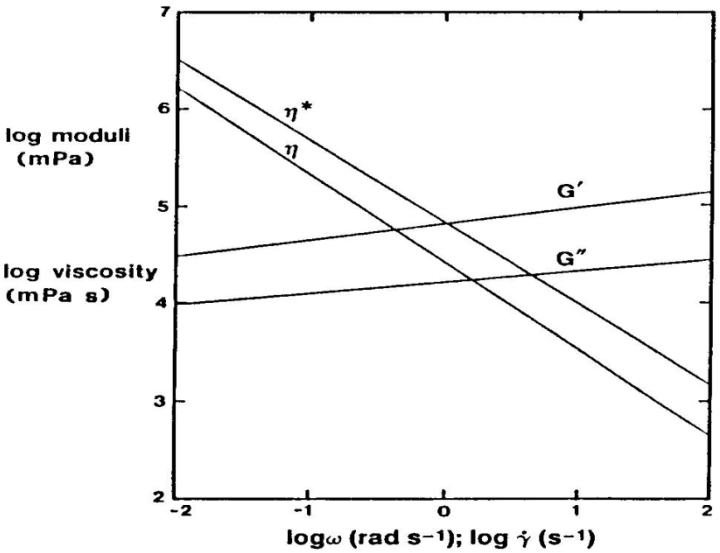


Fig. 3. Typical mechanical spectra for (a) polysaccharide gels and solutions of entangled coils, and (b) "weak gels" of ordered xanthan.

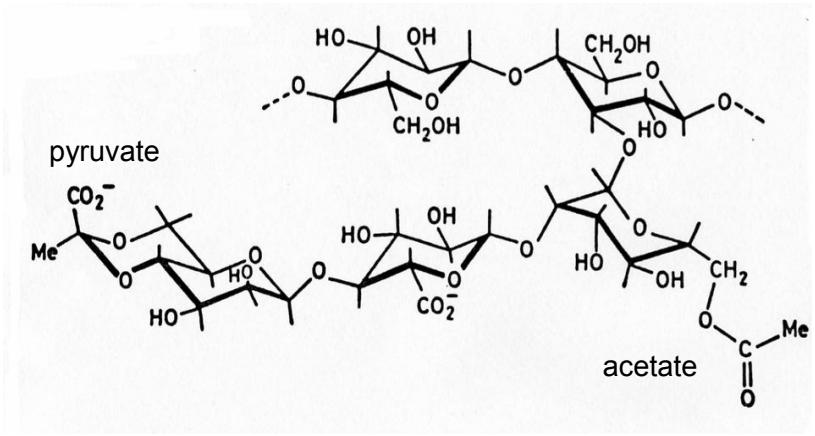


Fig. 4. Pentasaccharide repeat unit of xanthan (Jansson et al., 1975; Melton et al., 1976).

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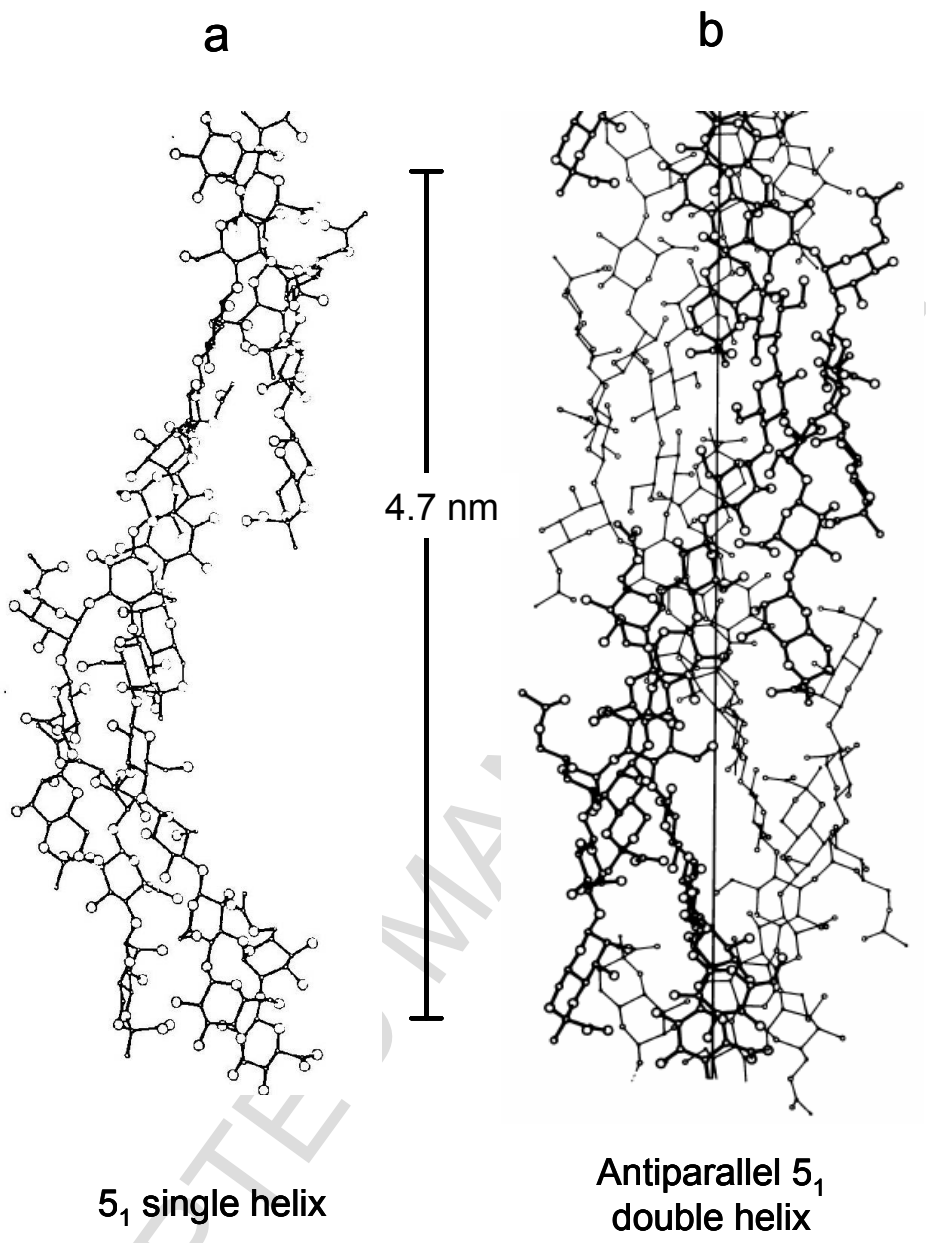


Fig. 5. Ordered structures proposed from computer-modelling of X-ray fibre diffraction data for xanthan. (a) Single helix (Moorhouse et al., 1977). (b) Antiparallel double helix (Okuyama et al., 1980). Both structures have 5-fold symmetry and a pitch of 4.7 nm, derived directly from the experimental diffraction patterns.

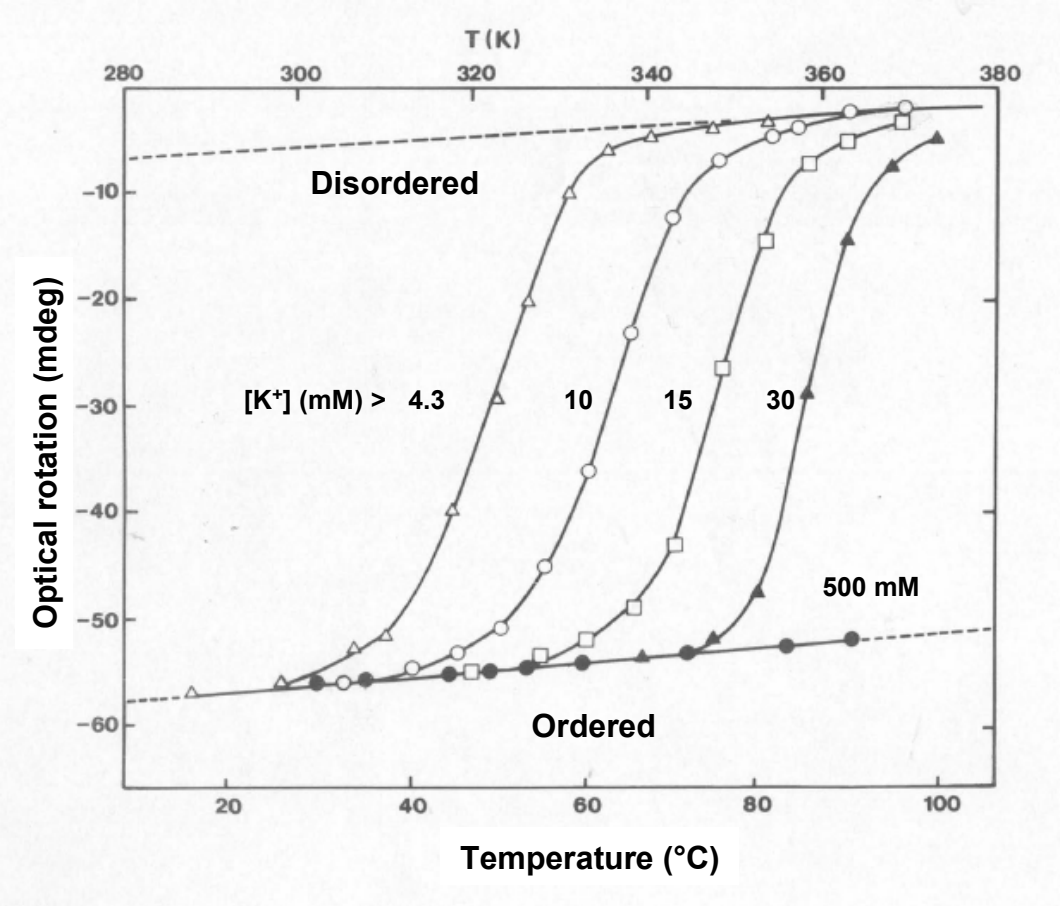
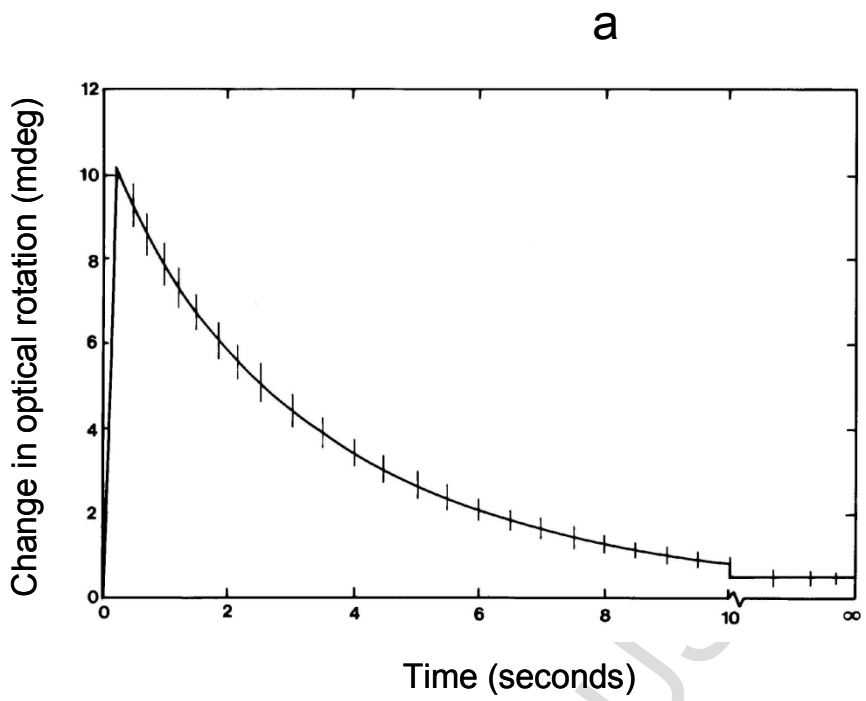


Fig. 6. Effect of salt (KCl) on the temperature-course of the conformational transition of xanthan, as monitored by changes in optical rotation (Norton et al., 1984).



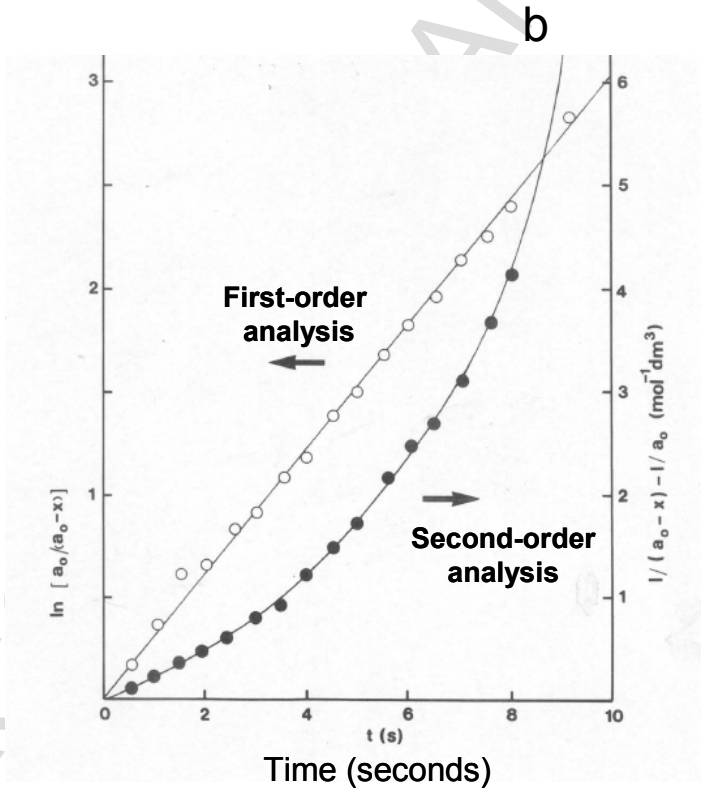


Fig. 7. (a) Typical reaction-progress curve for the salt-induced coil–helix transition of xanthan (Norton et al., 1984), illustrated for a mixture containing 0.5 mg/ml xanthan and 0.1 M KCl at 65°C; error bars show standard deviation of 25 traces. (b) Analysis of rate of change in optical rotation by first-order and second-order kinetic schemes.

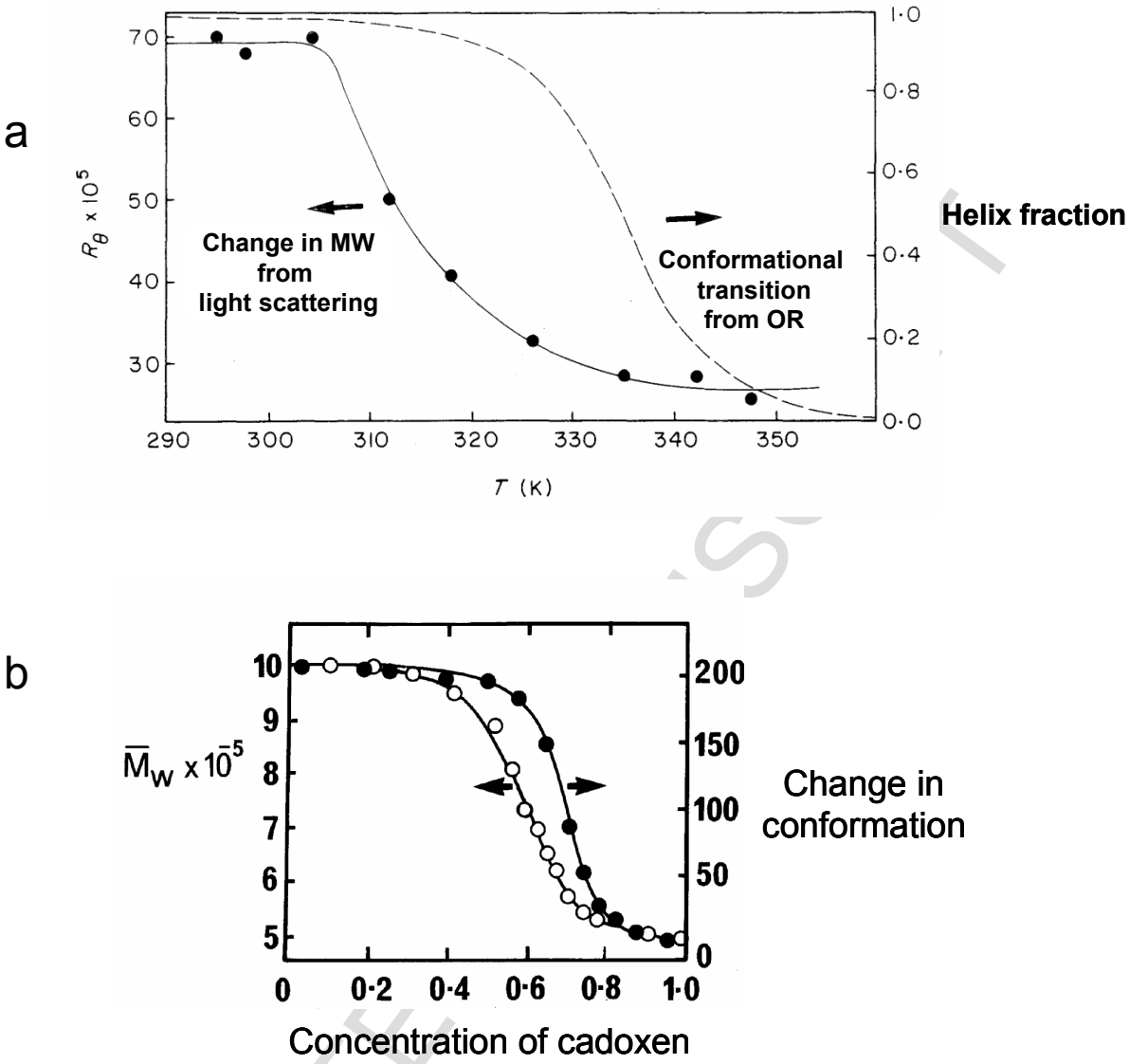


Fig. 8. (a) Comparison (Norton et al., 1984) of the temperature-course of changes in molecular weight from low-angle laser light scattering (left-hand axis) and the fraction of ordered structure, monitored by optical rotation (right-hand axis), for the same solution of xanthan (1 mg/ml in 10 mM KCl). (b) Effect of cadoxen concentration on molecular weight (left-hand axis) and conformation (right-hand axis) of xanthan (re-plotted from Kitagawa et al., 1985).

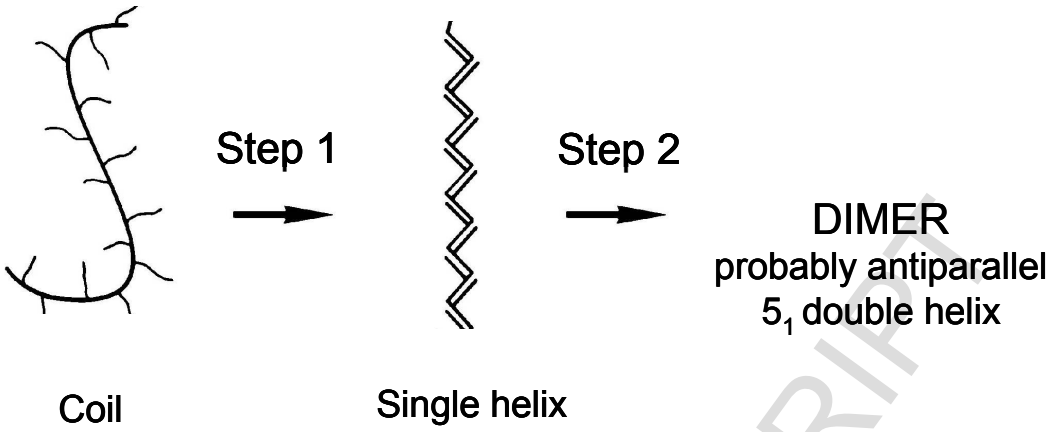


Fig. 9. Schematic illustration of the proposed mechanism of conformational ordering of xanthan.

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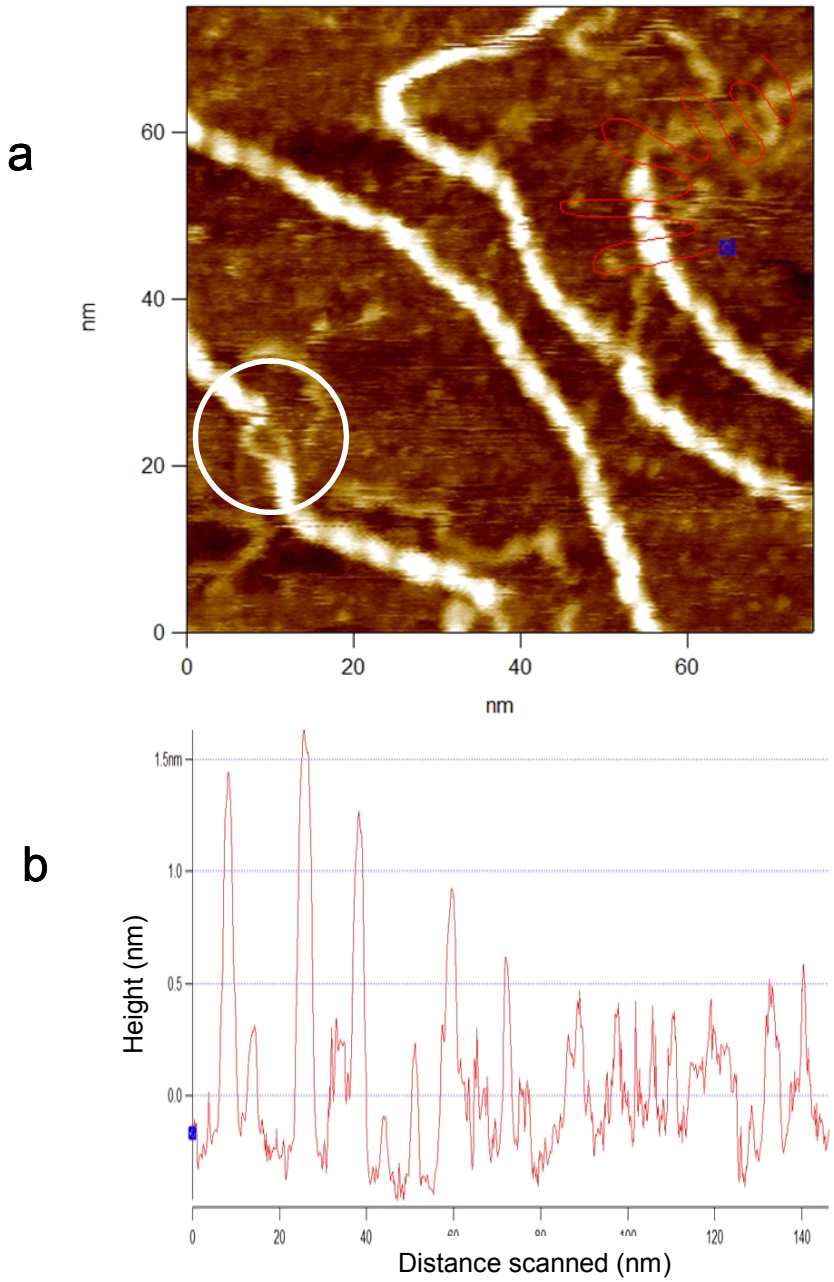


Fig. 10. (a) AFM image of xanthan recorded by Moffat et al. (2016). The feature circled shows unravelling of a short stretch of ordered structure into two strands. Separation into two disordered chains at the end of an ordered sequence can be seen at the top right-hand corner of the image. (b) Heights measured on going from ordered to disordered regions along the zig-zag marked in the top right-hand corner of frame a.