Carbohydrate Research, 160 (1987) 411–423
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

X-RAY FIBRE-DIFFRACTION STUDIES OF SYNERGISTIC, BINARY POLYSACCHARIDE GELS

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(Received March 12th, 1986; accepted for publication, July 5th, 1986)

ABSTRACT

X-Ray fibre-diffraction studies have been used to examine the proposal that intermolecular binding occurs between different polysaccharides in certain synergistic binary gels. No evidence for intermolecular binding was found in studies of tara-kappa carrageenan, carob-kappa carrageenan, tara-furcellaran, or carob-furcellaran gels. Present experimental data suggest that the most likely model for such gels consists of a galactomannan solution contained within a carrageenan or furcellaran network. However, evidence for intermolecular binding was found in fibres prepared from tara-xanthan and carob-xanthan gels. Gelation has been taken to involve an interaction of the cellulosic backbone of xanthan and the mannan backbone of the galactomannan Models for the junction zones of the mixed polymer network are discussed.

INTRODUCTION

Synergistic polysaccharide–polysaccharide interactions are attractive commercially and enjoy widespread technological exploitation[1]. Expensive polymers may be replaced by cheaper alternative formulations, and mixtures may be used to generate new functionality or to manipulate texture and rheology. Polysaccharide mixtures occur naturally, and binary gels may be used as models for complex cellular structures[2] and have been suggested as models for the recognition step in certain host–pathogen interactions[3].

When two polysaccharides are mixed together and gelled, several types of gel structure may arise, depending upon the nature of the components, the rate and extent of polymer demixing, and the mechanism of gelation[4,5]. In order to describe or predict the properties of a mixed gel, it is convenient to identify first the type of gel structure. For two polysaccharides *A* and *B*, the simplest structure involves *A* forming a network which merely contains *B* (Fig. 1a). At least three types of gel structure can occur if both polysaccharides contribute to the network. If both poly-

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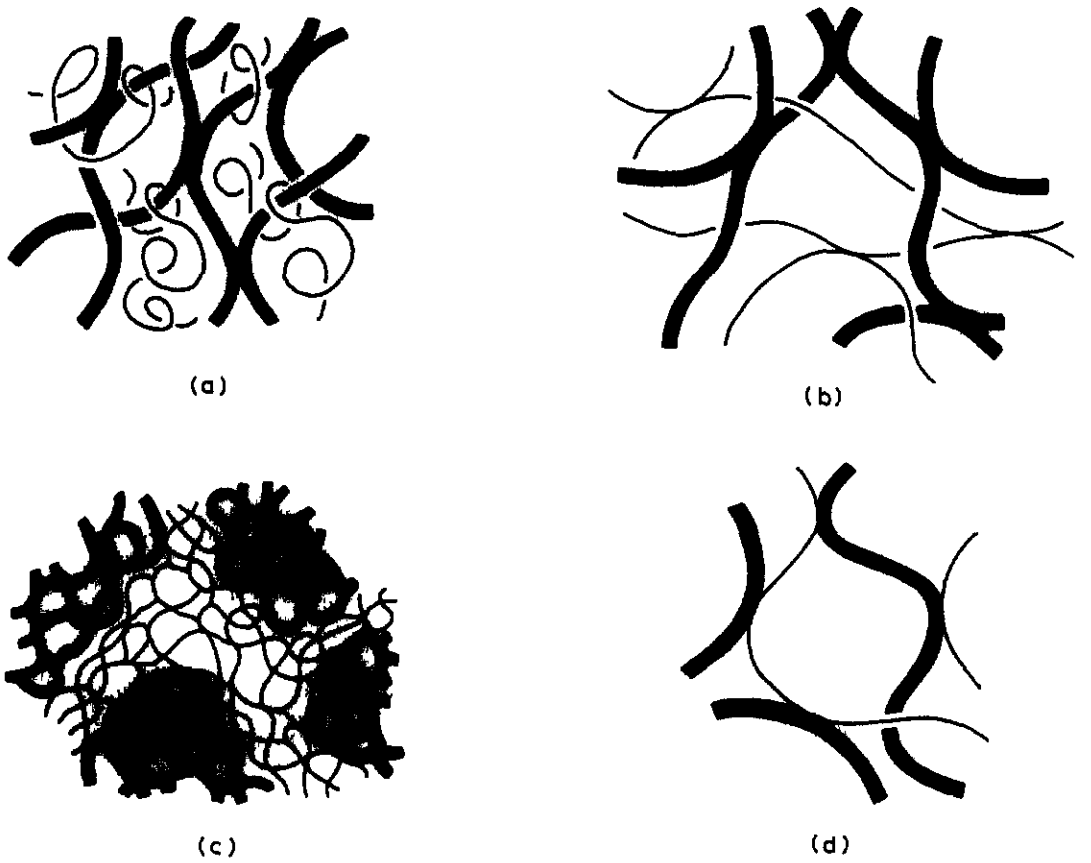

Fig 1 Types of binary polysaccharide gel-structure Polysaccharide *A* (■), polysaccharide *B* (—) (*a*) single polymer network containing the second polymer within the gel, (*b*) interpenetrating networks, (*c*) phase-separated networks, (*d*) coupled network

saccharides each associate independently to form separate networks which interlace, then the gel is an interpenetrating network (Fig. 1b). If some degree of demixing occurs prior to gelation, then the *A* and *B* networks will be separated spatially, resulting in a phase-separated network (Fig. 1c). Finally, if polysaccharide *A* binds to *B*, then the gel is a coupled network (Fig. 1d).

Synergistic interactions of polysaccharides in binary mixtures have often been considered to be synonymous with intermolecular binding of the two polysaccharides. The synergisms between plant galactomannans (carob, tara, or enzymically modified guar gum) and xanthan or certain algal polysaccharides (kappa-carrageenan, furcellaran, or agarose) have been attributed[2,3,6–8] to intermolecular binding of the backbone of the galactomannan and the helix of the other polysaccharide (Fig. 2). The evidence for this and for similar[9–11] well-accepted models is circumstantial and there is no direct proof of such intermolecular binding. Such binding is more likely to occur if the two polymers contain sequences which are structurally compatible. The structural similarity of galacturonic acid and guluronic acid blocks favours the formation of coupled networks between pectin and alginate. Comparative circular dichroism studies[12] of pectin, alginate, and pectin–alginate gels are suggestive of intermolecular binding, but the proposed models for intermolecular binding have not been tested. Binding between cellulose fibrils and the cellulosic backbone of xyloglucans has been suggested[13–15] as a natural coupled network in plant cell walls. Since mannose and glucose differ only in the orientation of HO-2, binding of the mannan backbone of galactomannans to cellulose fibrils is

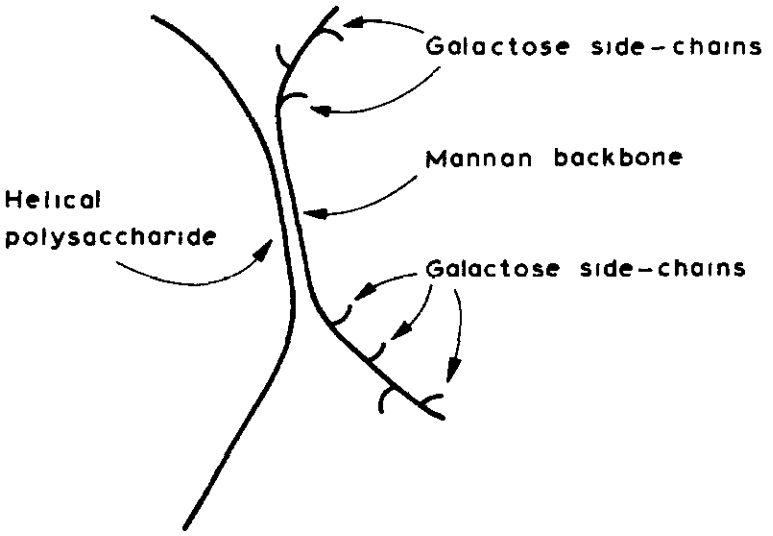

Fig 2 Suggested[2,3 6–8] model for the intermolecular binding of the bare mannan backbone of a galactomannan and the helix of certain algal polysaccharides (kappa-carrageenan, furcellaran, agarose) and the bacterial polysaccharide xanthan

sterically feasible, and cross-linking of cellulose fibrils by galactomannans has indeed been demonstrated by electron microscopy[16]. Xanthan–galactomannan binding (Fig. 2) has been proposed[3,6] as a recognition step in host–pathogen interactions.

Coupled networks are the only type of binary gel (Fig. 1) which contain *AB* junction zones. The technique of X-ray fibre diffraction may be used to test whether a binary gel contains *AB*, *AA*, or *BB* junction zones. This technique is the only method available to examine molecular models for gelation at atomic resolution. Although the preparation of fibres requires that the gels be stretched and at least partially dehydrated, studies of single polysaccharide systems have shown that, in all but one instance, the method provides reliable models for the ordered polysaccharide structures present in the hydrated state[17]. We now report X-ray fibre-diffraction studies of galactomannan–algal polysaccharide and galactomannan–xanthan binary gels. In those instances where evidence favouring the formation of a coupled network has been obtained, the X-ray data have been analysed to derive a model for the mixed-polymer junction zones. In those cases where no evidence for intermolecular binding has been found, additional experimental methods have been used to determine the type of network structure.

EXPERIMENTAL

Mannose and galactose contents of the galactomannans were determined by hydrolysis, conversion into the alditol acetates, and separation by g.l.c The mannose–galactose (MG) ratios were 3.55 (carob) and 3 (tara). The kappa-carrageenan was a mixed salt containing 2.75% of K^+, 0.53% of Na^+, and 1.61% of Ca^{2+} Characterisation of the carrageenan sample and the preparation of pure cation salts have been described elsewhere[18]. The potassium salt of furcellaran, prepared and purified as described elsewhere[19], was a gift from FMC Denmark. Xanthan was obtained from Sigma Chemicals. Fluorescent derivatives of tara and carob were also prepared[20]

Mixed biopolymer gels were prepared by mixing appropriate volumes of hot (95°) solutions of polysaccharide, pouring into moulds, and allowing the samples to cool to room temperature. Fibres for X-ray studies were prepared by cutting these gels into strips which were then stretched under conditions of controlled relative humidity (r h) and temperature. Compositions which did not gel were poured onto Teflon or glass substrates and partially dried to form films. Oriented fibres were prepared from these films X-Ray data were recorded photographically. The interior of the camera was maintained at a controlled r h. and was flushed with helium to reduce background scattering. CuK_α radiation (wavelength, 0 154 nm) was used and calcite was dusted onto the fibres for calibration. Elemental mapping of the samples was performed with a Philips PSEM 501B and cryo-sputter unit linked to a Link 860 series 2 EDS system Optical rotation data were obtained with an AA-100 polarimeter (Optical Activity Ltd).

RESULTS AND DISCUSSION

Pure components. — X-Ray fibre patterns obtained for carob and tara are shown in Fig. 3a–c Under the conditions used to study fibres prepared from mixed gels, the diffraction patterns obtained for carob (Fig. 3a) are poor. These patterns improve after annealing or storage of aligned fibres (Fig. 3b), owing to crystallisation of the galactomannan. The present diffraction patterns for both galactomannans (Fig 3a–c) are consistent with data reported elsewhere[16,21,22] Interpretation of the present data in terms of the proposed[16] unit cell gave carob [a = 3 17, b = 0.88, c (fibre axis) = 1.04 nm] and tara [a = 2.30, b = 0 81, c (fibre axis) = 1.04 nm]. The diffraction patterns obtained for kappa-carrageenan are sensitive to the cation content of the sample The lithium and sodium salts gave extremely poor fibre-diffraction patterns. Potassium and calcium carrageenates gave diffraction patterns with the first meridional reflection on the sixth layer line (Fig. 3d,e), whereas rubidium and caesium carrageenates showed meridional reflections on the third and sixth layer lines The layer line-spacing was consistent with data reported elsewhere[23,24]. Potassium furcellaran gave fibre diffraction patterns characteristic of potassium kappa-carrageenate except that meridional reflections were present on both the third and sixth layer lines X-ray fibre patterns (Fig. 3f) obtained for xanthan gum are consistent with reported data[16] that have been interpreted as a five-fold helix (pitch, 4.7 nm) with poor lateral packing of the helices. It has still not been determined whether xanthan adopts a single- or double-helical structure[25–28]

Galactomannan–algal polysaccharide gels. — Typical X-ray fibre diffraction patterns obtained for carob-kappa carrageenan and tara-kappa carrageenan gels are shown in Fig. 4. As reported previously[29–32], for all compositions studied, the X-ray fibre patterns obtained for the mixed gels show the characteristic patterns obtained for potassium and calcium kappa-carrageenate (Fig. 3d,e). The kappa-carrageenan used to prepare the mixed gels contains sodium, potassium, and cal-

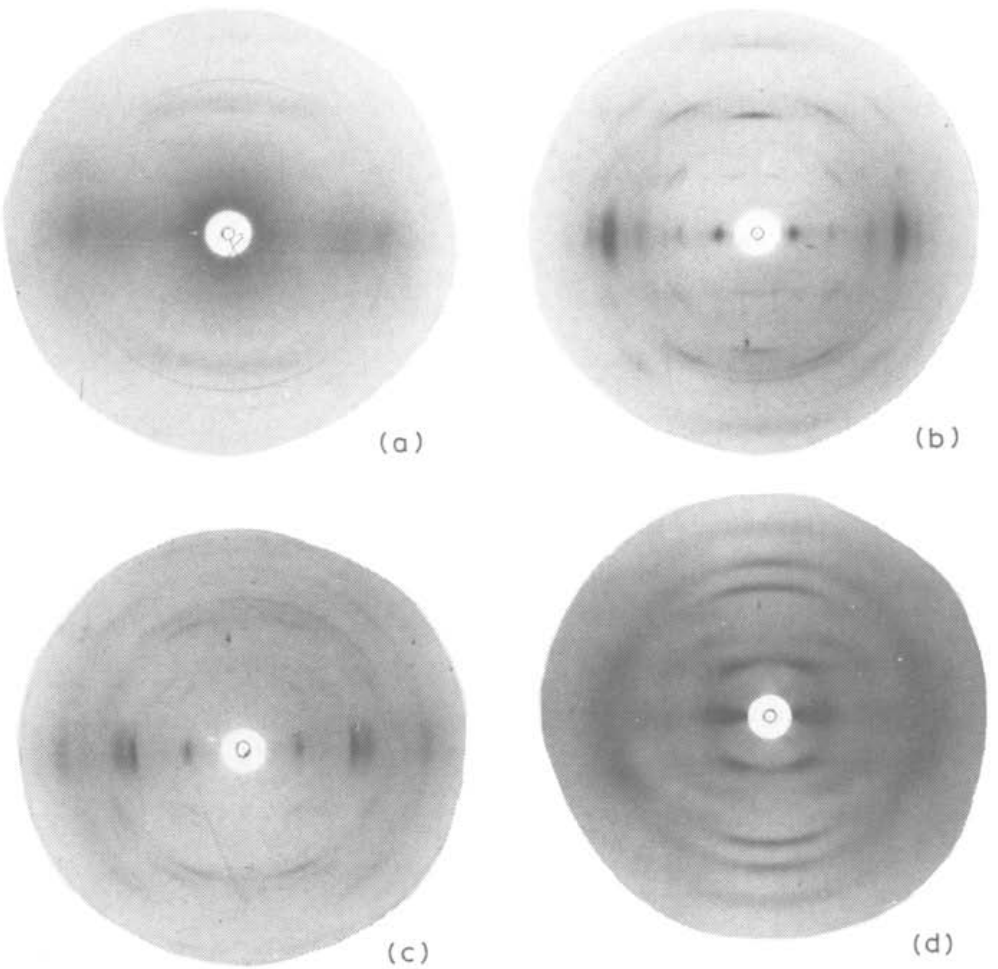

Fig 3 X-Ray fibre-diffraction patterns obtained for pure polysaccharides (wavelength, 0 154 nm, r h ~98%) (*a*) carob-stretched ~300%, (*b*) carob-stretched 300%, stored ~3 years at 20°, (*c*) tara-stretched 200%, (*d*) K^+ kappa-carrageenate, stretched 50%, (*e*) Ca^{2+} kappa-carrageenate, stretched 50%, (*f*) xanthan, stretched 300%

cium. Since sodium kappa-carrageenate yields very poor X-ray diffraction patterns, then, within experimental accuracy, the mixed-gel fibre-diffraction patterns may be considered to arise solely from the carrageenan component of the mixed gel. The size and type of unit cell is unchanged upon addition of galactomannan. There is no evidence to suggest that galactomannans are incorporated into the carrageenan junction zones of the gel network or that the galactomannans align or crystallise when the gels are stretched. Thus, it has not proved possible to detect the proposed intermolecular binding of the galactomannan and the carrageenan (Fig. 2). Similar results have been obtained for fibres prepared from furcellaran–galactomannan

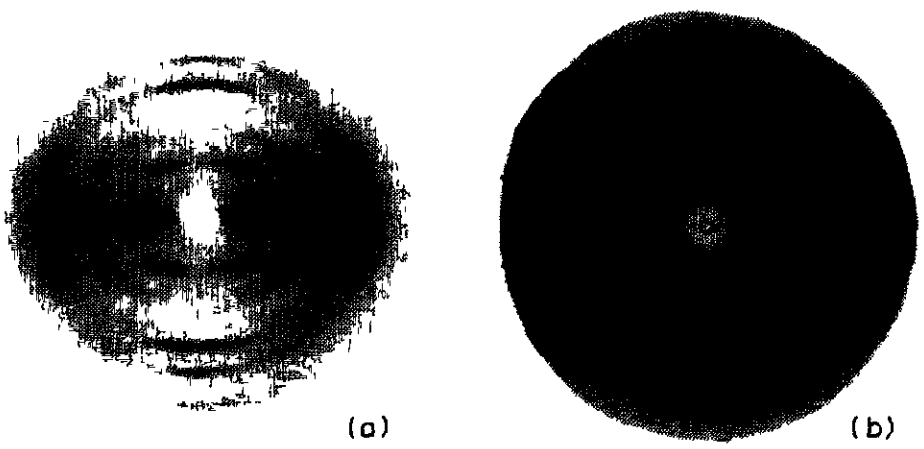

Fig 4 X-Ray fibre-diffraction patterns obtained for binary polysaccharide gels: (*a*) carob(50%)–kappa carrageenan(50%) gel, stretched 300%, (*b*) tara(50%)–kappa carrageenan(50%) gel, stretched 100% Wavelength, 0 154 nm, r h ~98%

mixed gels and will be reported elsewhere. The X-ray patterns obtained for fibres prepared from furcellaran–carob and furcellaran–tara mixed gels show the characteristic pattern obtained for fibres prepared from furcellaran alone. Thus, once again, it has not been possible to detect intermolecular binding of furcellaran and galactomannan.

In the absence of experimental data for the formation of coupled networks, further experiments were carried out to determine the orientation and spatial location of the two polysaccharides within mixed gels in order to identify the type of network structure. Information on the orientation of the galactomannans may be obtained by crystallising the galactomannans within aligned fibres prepared from mixed gels and noting the change in the X-ray pattern. Dehydration of tara-kappa carrageenan fibres did not lead to crystallisation of the tara gum or co-crystallisation of tara and kappa carrageenan[32]. It was observed that the crystallisation of carob fibres increased upon storage (Fig. 3a,b). Stored carob-kappa carrageenan fibres gave diffraction patterns showing a series of diffraction rings, due to carob crystallisation, superimposed upon the aligned fibre pattern characteristic of kappa-carrageenan junction zones. These data showed no evidence for co-crystallisation of carob and kappa-carrageenan, and demonstrated that carob was randomly oriented within the gel There are no standard methods for visualising individual polysaccharides within mixed gels In order to determine the spatial location of the two polymers within the gel, it is necessary to use natural or added labels to mark each polysaccharide Recent studies[33–35] suggest site binding of potassium within the junction zones of a pure kappa-carrageenan gel and, since kappa-carrageenan is sulphated, potassium and sulphur may be used to locate the carrageenan. Potassium and sulphur maps of mixed gels were obtained by rapidly quenching gels to the temperature of liquid nitrogen, transferring the gels to a scanning electron microscope, and recording the characteristic X-ray emission of each element resulting from electron bombardment upon scanning the gels. At a resolution of ~1 μm, potassium and sulphur mapping suggested uniform distribution of carrageenan within the gel. Fluorescent labelling of kappa-carrageenan affected the conforma-

tional transition and inhibited gelation, but labelling of carob and tara with fluorescein did not interfere with gelation. Tara-kappa carrageenan gels containing fluorescein-labelled tara were examined by fluorescent microscopy. At a resolution of ~1 μm, the tara gum appears to be uniformly distributed within the gel.

The simplest interpretation of the combined experimental data suggests that carob-kappa carrageenan, tara-kappa carrageenan, carob-furcellaran, and tara-furcellaran gels have structures of the type illustrated in Fig. 1a. This may account for the fact that, at least for a limited range of concentrations, the fracture stress and the elastic modulus of the mixed gels may be normalised with respect to the equivalent properties of a carrageenan gel having the same total polymer concentration, resulting in master curves characteristic of the type of galactomannan but independent of the total polymer concentration[36].

It could be argued that phase separation may occur but that the domains are less than 1 μm in diameter. However, in the absence of experimental evidence for phase separation, it will be assumed, within the limits of experimental resolution specified above, that the distribution of both polysaccharides is homogeneous. Proponents of an intermolecular-binding model might propose modifications of the model shown in Fig. 2 which may accommodate the present results. Thus, it could be suggested that the mixed gels contain large aggregates or microcrystalline carrageenan regions linked by surface attachment of galactomannan molecules. It would also be necessary to suppose that such interactions involve no preferential alignment of the surface-attached galactomannan, that the mannan attachment regions are small, or that they are dynamic rather than static linkages. However, in the absence of experimental evidence for such binding, and in view of the lack of steric compatibility between the mannan backbone and the carrageenan helix, the present experimental data favour a structure of the type shown in Fig. 1a

Galactomannan-xanthan gels — Typical X-ray diffraction patterns obtained for fibres prepared from carob-xanthan and tara-xanthan mixed gels are shown in Fig. 5. A comparison of Figs. 3a,b,c,f and 5a,b shows that the fibres prepared from

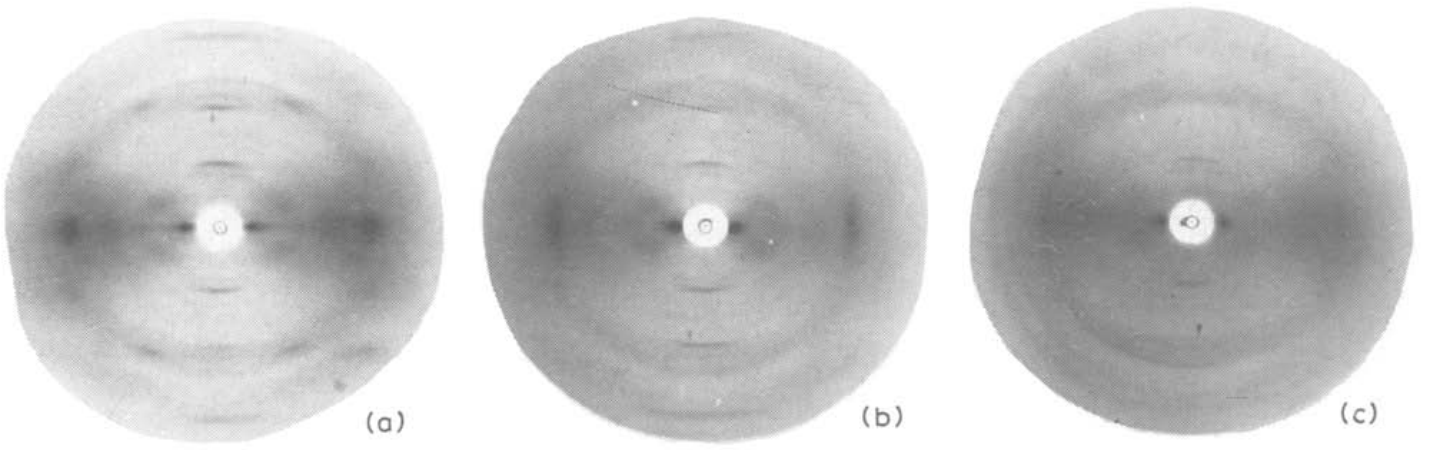

Fig 5 X-Ray fibre-diffraction patterns obtained for binary polysaccharide gels (*a*) carob(50%)–xanthan(50%) gel, stretched 300%, (*b*) tara(50%)–xanthan(50%) gel, stretched 300%, (*c*) carob(50%)–xanthan(50%) mixture prepared at room temperature, stretched 100% Wavelength, 0 154 nm, r h ~98%

the mixed gels yield new X-ray fibre patterns providing experimental evidence for xanthan–galactomannan binding. A preliminary account of such studies has been reported[37]. The data shown in Fig. 5a,b provide a basis for a quantitative analysis, at atomic resolution, of models for the xanthan–galactomannan interaction However, X-ray fibre patterns alone will, in general, contain insufficient information to generate a complete molecular description of the interaction, and must be used to assess the merits of models proposed upon the basis of other experimental evidence. In view of the complexity of such modelling procedures, the following experiments were carried out in order to ascertain the main features of the intermolecular interaction.

The model shown in Fig. 2 has been proposed[3,6] to account for xanthan–galactomannan binding. This model proposes cooperative interaction in which the ordered helical structure of the xanthan is retained. The stoichiometry of the interaction is undefined and there is no obvious steric compatibility between the mannan backbone of the galactomannan and the xanthan helix Thus, it is not possible sterically to construct in three dimensions a model compatible with Fig 2 In view of these difficulties, experiments have been designed to test the central postulate of the model that the xanthan helix is retained within the mixed junction zone of the two polysaccharides.

The following mixing experiments were carried out in order to test whether the formation of a xanthan helix is essential for xanthan–galactomannan binding and hence gelation. Samples of xanthan and carob were separately dispersed in water at 95° and each sample cooled to room temperature. Optical rotation studies, which are sensitive to the helix–coil transition[27], were used to demonstrate that xanthan adopted the helical form. Thorough mixing of these two samples at room temperature did not lead to gelation. X-Ray diffraction patterns obtained for fibres prepared from such mixtures were poor (Fig. 5c) but showed reflections characteristic of xanthan alone (Fig. 3f). When these mixtures were heated to 95°, a temperature above the xanthan helix–coil transition temperature (T_c), and then recooled to room temperature, they gelled Fibres prepared from these gels gave new X-ray fibre diffraction patterns (Fig 5a). Clearly, the mixtures need to be heated to induce gelation In order to determine whether heating merely enhanced mixing or was necessary to denature the xanthan helix, an analogous experiment was performed in which the xanthan and carob were mixed at high temperatures, but with xanthan in the helical rather than coil form. The transition temperature (T_c) is sensitive to ionic strength[38]. Sufficient quantities of calcium chloride were added to the xanthan solution to raise T_c above 100°. When such xanthan–carob mixtures containing calcium chloride were heated to 95° and then recooled to room temperature, they did not gel These experiments suggest that a central postulate of the model depicted in Fig 2, namely, retention of the xanthan helix upon binding, is incorrect and indicate that the xanthan helix must be denatured if intermolecular binding is to occur.

The primary structure of xanthan is a (1→4)-linked β-D-glucan backbone

(cellulose) substituted through O-3 on alternate glucosyl residues with a charged trisaccharide side-chain[39,40]. Galactomannans consist of a mannan backbone incompletely substituted at O-6 by galactose side-chains[7]. Glucose and mannose differ only in the orientation of HO-2. Thus, the interaction of the cellulosic and mannan backbones provides a stereochemically acceptable basis for xanthan–galactomannan binding.

The following arguments have been used to reduce further the number of possible models for the intermolecular interaction. Studies[41,42] of the action of β-D-mannanase on carob in the presence of xanthan demonstrated that xanthan inhibited the enzymic degradation only if the mixtures were preheated prior to addition of the enzyme. This is consistent with the view that it is necessary to denature the xanthan helix if intermolecular binding of xanthan and carob is to occur. Even in xanthan–carob mixtures which had been preheated, the degree of inhibition was small, suggesting the involvement of small segments of the galactomannan backbone[41,42]. Optical rotation studies[3] of xanthan–galactomannan mixed gels demonstrate substantial recovery of optical rotation on cooling and gelation. This suggests that, if the intermolecular binding involves the denatured xanthan, only small segments of the xanthan molecule are involved in binding and that the remainder of the molecule re-forms into the helical structure. Alignment of the junction zones within the mixed gels will cause alignment of the xanthan helices. Thus, the X-ray patterns obtained for fibres prepared from the mixed gels (Fig 5a,b) will contain xanthan diffraction patterns superimposed on the pattern due to the mixed-polymer junction zones. In the remainder of this discussion, only these reflections in mixed-gel patterns (Fig. 5a,b), which are not attributable to xanthan alone, will be considered. The position of the first meridional reflection corresponds to an interplanar spacing of 0.52 nm, which is equivalent to the axial advance per repeat unit characteristic of cellulose[43] and mannan[44]. Simple binding schemes involving units of the type shown in Fig 6a,b,c would be expected to give an axial advance per chemical repeat of 1.04 nm. A sandwich structure (Fig. 6d), in which the relative positions of the xanthan side-chains are staggered, could explain the observed repeat distance of 0.52 nm. The exact stoichiometry of the junction zones is not known and several galactomannan molecules might be sandwiched between xanthan backbones. The interior of the sandwich may need to accommodate galactose-substituted as well as bare mannan backbones Certain limitations may be placed on the possible models if the diffraction patterns shown in Fig 5a,b are compared with those of the pure galactomannans shown in Fig. 3b,c. The mixed-gel diffraction patterns are equivalent to pure galactomannan patterns for which only 0kl reflections are allowed. A simple explanation would be to consider that the sandwich structure may grow in the *c*- and *b*-dimensions, but that the surface coatings of xanthan poison growth in the *a*-dimension. Such a micellar structure, with the hydrophilic xanthan side-chains immersed in the aqueous environment and the hydrophobic cellulosic and mannan segments buried in the interior of the sandwich, is an attractive model. However, the streaking of reflections on all layer lines

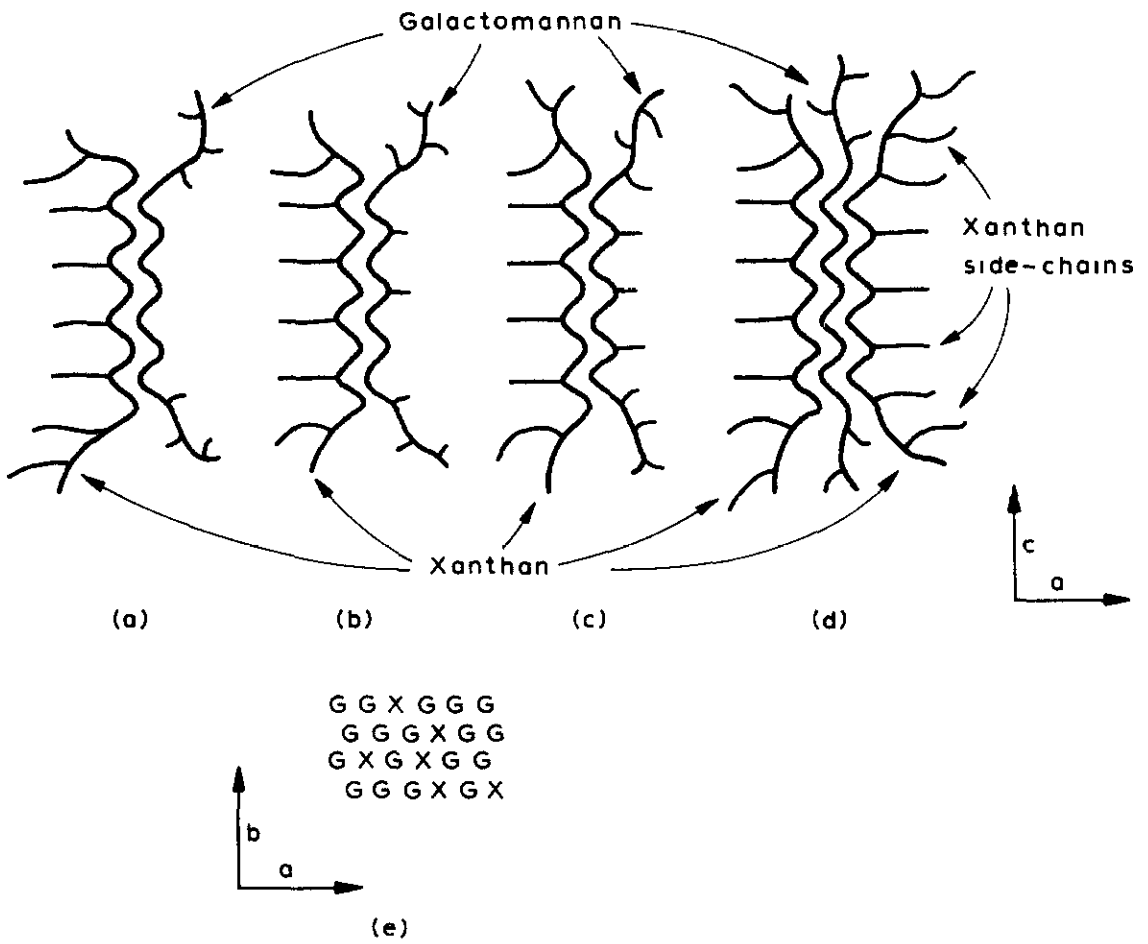

Fig 6 Possible types of galactomannan–xanthan binding Binding of a xanthan backbone with (*a*) bare mannan segment of galactomannan, (*b*) irregularly galactose-substituted mannan backbone, and (*c*) alternately galactose-substituted mannan backbone (*d*) Sandwich structure–bare mannan backbone sandwiched between two xanthan backbones (*e*) Random co-crystallisation of xanthan and galactomannan–schematic diagram of *a–b* plane The diagrams are schematic, illustrating the principle of the suggested binding

expected for a laminate structure was not observed experimentally (Fig. 5a,b). An alternative is to consider that the dimension in the *a*-direction is not small, but that the structure is aperiodic. The sandwich structure could be retained by allowing the xanthan to coat galactomannan crystallites in which irregular galactose substitution perturbs the *a*-dimension but not the *b*- or *c*-dimensions. However, the patterns shown in Fig 3b,c suggest that such substituents may be adequately accommodated within a galactomannan lattice. The next simplest alternative is to envisage a random co-crystallisation of the xanthan and galactomannan, resulting in a galactomannan lattice threaded by xanthan molecules (Fig. 6e). The trisaccharide side-chains of xanthan are larger than the galactose side-chains of the galactomannans and might be considered to act as defects in the *a*-dimension, leading to aperiodicity. The above analysis is qualitative but provides a basis for a subsequent quantitative analysis of models through a comparison of predicted diffraction patterns with the data shown in Fig. 5a,b

The present data show that xanthan–carob and xanthan–tara intermolecular binding can occur. Thus, gelation of such mixtures could involve the formation of coupled networks. The compatibility of the cellulosic and mannan backbones explains why galactomannans can bind to xanthan but not to kappa-carrageenan or furcellaran. The studies support suggested binding of xyloglucans[13–15] or galacto-

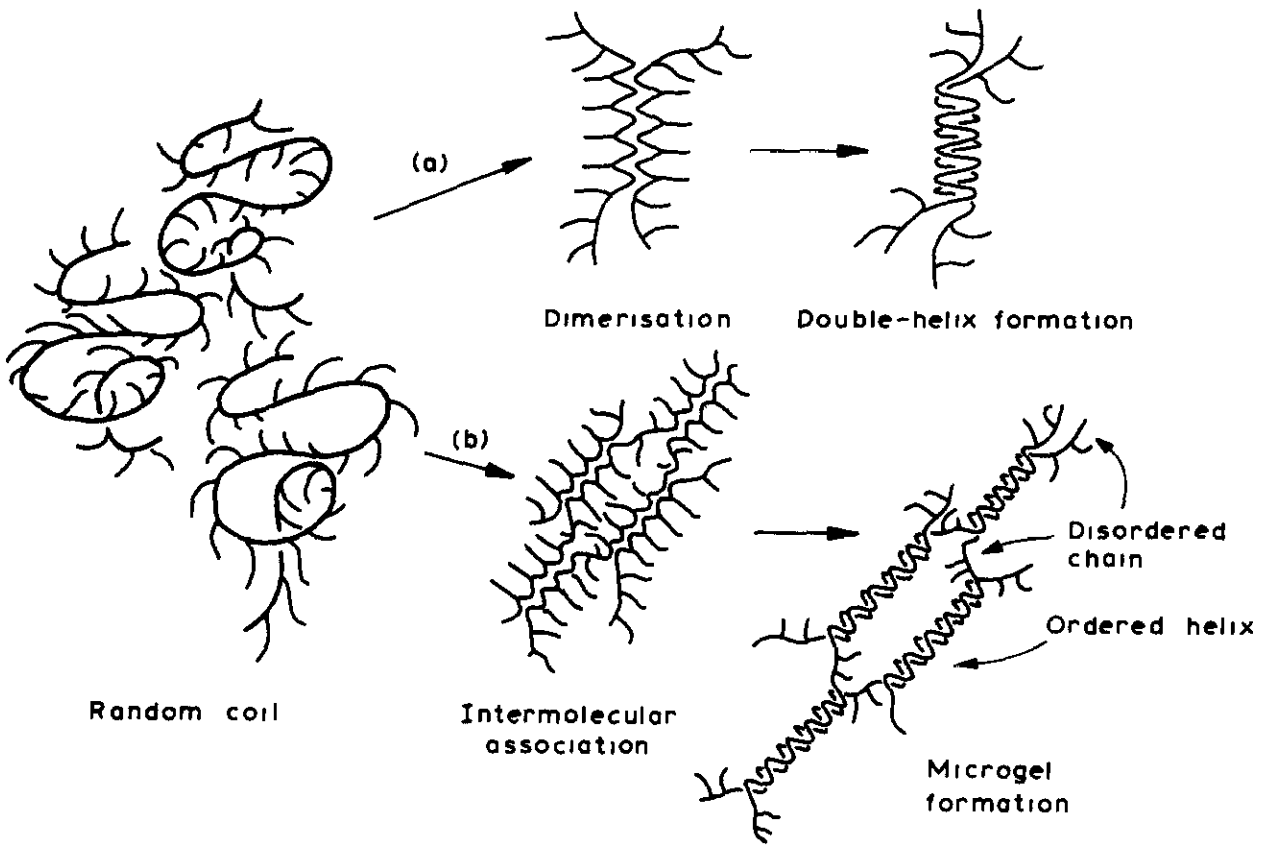

Fig 7 Molecular association in xanthan samples (*a*) Double-helix formation in dilute solution (i) random coils, (ii) association of backbones, (iii) twisting of backbone to form a double helix stabilised by binding of the side-chains to the backbone (*b*) Intermolecular association of several xanthan molecules leading to formation of a microgel

mannans[16] with cellulose. The binding of extracellular xanthan to plant cell-wall components, suggested as a host–pathogen recognition step and to account for binding within plant vascular systems, will only arise if the xanthan is in the non-helical form. Such extracellular assemblage cannot be ruled out because such processes are normally controlled biochemically. Xanthan–galactomannan interactions are known to be sensitive to the MG ratio of the galactomannan[7]. An explanation of this effect will require a more detailed picture of the mixed-polymer junction zone

If the xanthan backbone can interact with the galactomannan backbone, it is surprising that the xanthan backbone does not bind to other xanthan backbones. Xanthan does not gel, but does form aggregates or microgels. The xanthan sidearms would tend to inhibit association beyond dimerisation (Fig. 7a). Parallel pairing of cellulosic backbones would lead to an axial rise per individual glucosyl residue within each chain of 0.52 nm. A slight twist of the paired backbones, presumably stabilised by side-chain–backbone binding, could reduce this axial repeat distance from 0.52 to 0.47 nm. This structure could possess a periodicity consistent with the observed layer-line spacing of 4 7 nm, but the staggering of the positions of the xanthan side-chains would result in a meridional reflection on the tenth layer line but no meridional reflection on the fifth layer line. Double-helix formation may be preferable to molecular association beyond dimerisation. The poor quality of the fibre pattern shown in Fig. 3f and that reported elsewhere[25,26] make it difficult to be certain that the first meridional reflection does arise on the fifth layer line A

double helix based on a modified pairing of the cellulosic backbones is worthy of further investigation since simultaneous intermolecular association of several chains (Fig. 7b) under appropriate conditions could explain the formation of xanthan microgels and account for many unusual features of the rheology of xanthan samples.

ACKNOWLEDGMENTS

The authors thank Dr. C. Blood for the gift of the tara gum, and FMC Denmark for preparing the sample of furcellaran. V. Carroll, G. R. Chilvers, M. A. O'Neill, M. Ridout, and R. Turner are thanked for experimental assistance, and Professor E. D. T. Atkins is thanked for helpful discussions.

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