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Affinity Interactions Between Agarose and β-1,4-Glycans: a Model for Polysaccharide Associations in Algal Cell Walls

Iain C. M. Dea and David A. Rees*

Unilever Research, Colworth Laboratory, Sharnbrook, Bedford MK44 1LQ, UK

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SUMMARY

This paper reviews the extensive and previously unpublished work on the interactions between agarose and 1,4-linked β-D-glycans carried out at Unilever Research, Colworth Laboratory, UK. The effect of the following variables is discussed: (i) galactose content of galactomannans; (ii) substitution patterns in the agarose molecule; (iii) structural variations in the 1,4-β-D-glycan main chain; and (iv) molecular size of the 1,4-β-D-glycans.

Double helices of agarose, a non-substituted regular polysaccharide comprising 1,3-linked β-D-galactose and 1,4-linked 3,6-anhydro-α-L-galactose, bind in an ordered cooperative fashion to an extended ribbon ordered conformation of sequences of 1,4-linked β-D-mannopyranose residues in plant galactomannans to give mixed gelling systems. This interaction survives, in a modified form, substitution along the agarose molecule by O-methyl ether and O-sulphate esters at O6 of the D-galactose and O2 of the 3,6-anhydro-L-galactose, and 4,6-linked pyruvic acid ketal groups on the D-galactose. The higher the level of substitution on the agarose, the weaker the interaction with galactomannan.

In general, the higher the level of galactose substitution in the galactomannan the lower the extent of interaction with agarose. Evidence is presented, however, which indicates that the fine structural distribution of galactose along the galactomannan molecule is also an important determinant for the co-gelling interaction. Substituted 1,4-linked β-D-glucomannans, β-D-glucans and β-D-xylans which can form closely similar extended ribbon order conformations to the galactomannans also participate in co-gelling interactions with agarose. These β-D-glycans are

*Present address: National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK.

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similar in structure to important skeletal polysaccharides such as hemicelluloses and cellulose. This suggests that the binding between agars and β-D-glycans might mimic biological cohesion between skeleton and gel phases in natural red seaweed cell walls. The sensitivity of the interactions studied to fine details of agar and β-D-glycan structure is what might be expected on biological grounds, since the wide and subtle variations of natural polysaccharide structure are presumed to represent a mechanism for control of their intermolecular interactions.

INTRODUCTION

Cellulose constitutes the main skeletal polysaccharide in most plant cell walls. A range of structurally related plant polysaccharides, however, exists which performs similar functions in the cell wall. These alternative skeletal polysaccharides can be divided into two groups. The first involves a change in configuration at C2 to give the glucomannans and mannans, while the second involves the loss of the C6 hydroxymethyl group to give the xylans. In addition, further variation can result from the substitution of acetate groups and sugar residues. In the latter case this results in the arabinoxylans and the arabino-glucuronoxylans. It has long been recognised that a gradation in degree of crystallinity, and hence strength of association and mechanical properties, exists within this series of plant skeletal polysaccharides (Marchessault & Sundararajan, 1976). However, this series of polysaccharides has not been found amenable to reconstitution studies *in vitro*; indeed even Cellulose I cannot be reconstituted.

Reconstitution studies have been carried out on simple polysaccharide gel systems which model the more highly hydrated phases of plant cell walls (Rees, 1981). These studies have shown the generality of interrupted periodic sequences which function by associating through ordered junction zones and linking through disordered segments of polysaccharide chains. In addition, it has been demonstrated that biosynthesis of these gelling structures often occurs through enzymic modification at the polymer level to generate sequences for association. Examples of such modifications in polysaccharide structure include the epimerisation of D-mannuronic acid to L-guluronic acid in the alginate series (Madgwick *et al.*, 1973) and the transformation of D-galactose-6-sulphate to 3,6-anhydro-D-galactose in the carrageenan series (Lawson & Rees, 1970).

It is possible that, in the plant cell wall, these hydrated phases may associate specifically with the skeletal polysaccharide phases, and the

polysaccharide structure modification in both the hydrated and skeletal phases may influence such interactions. Our earlier reports (Dea *et al.*, 1972; Dea, 1981) on the interaction between both agarose and the carrageenans with β-1,4-glycans suggested that these could be good models for reconstitution studies of polysaccharide associations in algal cell walls.

The gel forming polysaccharides agarose and the carrageenans (κ and ι) are regular alternating copolymers, containing varying amounts of sulphate ester, methyl ether and pyruvic acid ketal substituents. The structure of these polysaccharides comprises 1,3-linked β-D-galactose and either 1,4-linked 3,6-anhydro-α-L- or D-galactose for agarose and the carrageenans, respectively (Araki & Arai, 1967; Anderson *et al.*, 1968*a*,*b*; Duckworth *et al.*, 1971). It has been firmly established that agarose (Arnott *et al.*, 1974*a*), ι-carrageenan (Arnott *et al.*, 1974*b*) and probably also κ-carrageenan (Anderson *et al.*, 1969) can adopt ordered double helical conformations in the condensed phase. None of these polysaccharides consist entirely of regular, alternating sequences of sugar residues; interruptions of 'kinks' occur when the regular 3,6-anhydro galactose residues are replaced by galactose, and can normally be removed by a Smith degradation procedure to create short blocks or segments, which retain the ability to adopt ordered conformations but cannot gel (Rees, 1961). Both these segments and the parent polysaccharides show temperature dependent sigmoidal changes in optical rotation, the signs and magnitudes of which correspond closely to those predicted from the double helix geometry in the solid state, using semi-empirical calculations of optical activity from glycosidic angles of the polysaccharide backbone (Rees & Scott, 1971). In addition, in the case of ι-carrageenan segments, the optical rotation change shows a concentration dependence which corresponds to a dimerisation process (Bryce *et al.*, 1974), and this disorder to order transition is accompanied by an exact doubling in number-average and weight-average molecular weight (Jones *et al.*, 1973). For both κ-carrageenan and naturally sulphated agarose, the dynamics of salt-induced disorder transitions have been studied by a polarimetric stopped-flow technique (Norton *et al.*, 1979). The results indicate that the formation of the ordered state is a second-order process, and strongly indicate that the aggregates of order chains in the junction zones of these gels comprise double helical entities. The evidence is therefore compelling that, under favourable conditions of temperature, concentration, ionic strength and appropriate counter ions, the agarose and carrageenan double helices which exist in the solid state can survive in highly hydrated solutions and gels.

We have previously reported (Dea *et al.*, 1972) that the double helices of agarose and κ-carrageenan, but not ι-carrageenan, could bind in an ordered, co-operative fashion to unsubstituted sequences of 1,4-linked β-D-mannopyranose residues in certain plant galactomannans, and that such mixed polysaccharide systems can lead to unexpected and useful rheological properties. It was proposed that the binding mechanism involves non-covalent association between unlike polysaccharide chains in ordered, complementary conformations (a double helix and an extended ribbon). The stoichiometry of binding and the particle weight of the complex are not known, although the turbidity of the mixed systems suggests qualitatively that many or all of the subunits exist in aggregated form.

The initial study (Dea *et al.*, 1972) showed that the strength of the interaction between galactomannans and agarose or κ-carrageenan increases with decrease in galactose content. The same trend was observed for the gelling interaction between the extracellular polysaccharide from *Xanthanomas campestris* (xanthan) and galactomannans (Dea *et al.*, 1977; McCleary *et al.*, 1981). The co-gelling properties of xanthan and galactomannans have been interpreted as resulting from non-covalent association of the native conformation of xanthan with an extended ribbon conformation of the galactomannans. Recent studies have indicted that, in addition to galactose content of the galactomannan, fine structural differences in the distribution of galactose along the mannan backbone of the galactomannan molecule can also significantly affect the strength of the interaction with agarose and xanthan (McCleary, 1979; Dea *et al.*, 1986*a*, *b*). In addition, the interaction between xanthan and a lightly acetylated 1,4-linked β-D-glucomannan has been examined (Dea *et al.*, 1977). This β-D-glycan interacts much more strongly with xanthan than the galactomannans studied, both in terms of minimum gelling concentration and melting point of mixed gels. It is therefore clear that major modifications in interaction properties can result from changes in galactose content of galactomannans, fine structural differences between galactomannans and differences in the chemical nature of the 1,4-linked β-D-glycan.

The work reviewed in this paper extends the examination of this interaction between agarose and 1,4-linked β-D-glycans as a model for the biological assembly of algal cell walls. The sensitivity of the strength of these interactions to chemical variations such as sulphate level in agarose/carrageenan and structural differences between 1,4-linked β-D-glycans would not be unexpected on biological grounds, since chemical structure variation is presumed to represent a mechanism for controlling biological associations. It was therefore decided to examine in detail how

chemical structure variations affect the extent and strength of the interaction between agarose and 1,4-linked β-D-glycans. This has involved the study of a wide range of naturally substituted agars, a wide range of structurally related 1,4-linked β-D-glycans (D-mannans, D-glucomannans, D-glucans and D-xylans), and a preliminary investigation of the effect of lowering the molecular weight of the β-D-glycan.

MATERIALS AND METHODS

(a) Structural variants of algal galactans

(i) *Agarose*

The agarose used was a production batch (batch code REX 5468), obtained from Marine Colloids, Inc. (Rockland, Maine, USA). This sample was used in earlier investigations (Dea *et al.*, 1972; Arnott *et al.*, 1974*a*) and is similar in its behaviour to an agarose purified by column chromatography (Duckworth & Yaphe, 1971). The sample is essentially free of substituents.

(ii) *Natural 6-O-methylated agars*

A range of 16 natural *O*-methylated agars was obtained from Marine Colloids, Inc. The methoxyl contents are listed in Table 1. They had all been previously studied by Guiseley (1970), and the sample numbers used in Guiseley's study are also listed in Table 1.

(iii) *Porphyran*

The alkaline borohydride treated porphyran was provided by Dr J. R. Turvey. This galactan, obtained from *Porphyra umbilicalis*, is similar to agarose, but contains about one 6-*O*-methyl substituent for every two disaccharide residues (Duckworth, 1968).

(iv) *Natural 2-O-methylated agar*

The agar from *Rhodemela larix* was prepared as described by Usov *et al.* (1971), from algae collected by Dr J. N. C. Whyte in British Columbia, Canada. This agar is 2-*O*-methylated on about half of the 3,6-anhydro-L-galactose residues (Usov *et al.*, 1971).

(v) *Natural pyruvic acid derivative of agar*

The agar from *Gracilaria compressa* was used in an earlier investigation (Arnott *et al.*, 1974*a*). This agar contains 3% of combined pyruvic acid (Young *et al.*, 1971), which corresponds to the presence of D-galactose and 4,6-*O*-carboxyethylidene-D-galactose in the ratio 7:1.

TABLE 1
Natural *O*-Methylated Agars Obtained from Marine Colloids, Inc.

Sample number	*OCH_3 (%)*	*Sample number in the study of Guiseley (1970)*
1	0·43	3
2	0·72	5
3	0·89	9
4	1·07	11
5	1·63	15
6	1·91	48
7	2·03	21
8	2·26	25
9	2·37	47
10	2·53	26
11	3·00	40
12	3·47	39
13	4·58	42
14	5·04	43
15	6·59	45

(vi) *Natural agarose sulphate*
The alkaline borohydride treated agar from *Gloeopeltis cervicornis* was that used in an earlier investigation (Arnott *et al.*, 1974*a*). All the D-galactose residues in this polysaccharide are 6-*O*-sulphated and 20% of the 3,6-anhydro-L-galactose residues are 2-*O*-sulphated (Penman & Rees, 1973).

(vii) *Segmented agarose*
Low molecular weight agarose segments were prepared by carrying out a Smith degradation (Goldstein *et al.*, 1965) on agarose, using the procedure described earlier (Dea *et al.*, 1972).

(viii) *Carrageenans*
κ-Carrageenan from *Chrondrus crispus* was obtained from Marine Colloids, Inc. (batch code REX 5401). The sample is not an ideal κ-carrageenan but contains a high percentage (30–35%) of the 3,6-anhydro-D-galactose residues as the 2-sulphate. The material was used in the potassium salt form. ι-Carrageenan from *Eucheuma spinosum* was the same sample as used in earlier work (Arnott *et al.*, 1974*b*). The carrageenan from *Furcellaria fastigiata* (furcellaran) was obtained from Marine Colloids, Inc. Furcellaran is similar to κ-carrageenan except that only half the D-galactose residues are sulphated at C4 (Yaphe, 1959).

(b) 1,4-Linked β-D-glycan variants

(i) *Native plant seed galactomannans*

The galactomannan from seeds of *Trigonella foenum-graecum* was extracted by the method of Andrews *et al.* (1952). The seeds were obtained from Marine Colloids, Inc. Guar gum from *Cyamopsis tetragonolobus* was a purified grade purchased from Kobenhavns Pektinfabrik (Copenhagen, Denmark). Two samples of galactomannan from *Gleditsia triacanthos* (Leschziner & Cerezo, 1970) were donated by Dr A. S. Cerezo. Sample 1 was a cold water extract of whole seeds, and sample 2 was a 50–60°C extract of whole seeds. Tara gum from *Caesalpinia spinosa* was purified material obtained from Marine Colloids, Inc. (batch code REX 5922). The galactomannan from *Gleditsia amorphoides* (Cerezo, 1965) was also donated by Dr A. S. Cerezo. The galactomannan from *Caesalpinia pulcherima* (Unrau & Choy, 1970*a*) was a gift from Professor A. M. Unrau. Locust bean gum from *Ceratonia siliqua* was a purified grade purchased from Kobenhavns Pektinfabrik (Copenhagen, Denmark). Analysis of these eight samples indicated the galactose contents listed in Table 2.

The galactomannan from seeds of *Crotalaria mucronata* was extracted by the method of Unrau & Choy (1970*b*). The seeds were obtained from Professor A. M. Unrau. Analysis indicated a composition of 73% mannose and 27% galactose. The galactomannan from *Crotalaria mucronata* differs structurally from the other galactomannans studied in having only 78% of the residues in the main chain linked β-1,4, the remainder being either 1,2- or 1,3-linked.

(ii) *Fractionated* Ceratonia siliqua *galactomannan*

The galactomannan from *Ceratonia siliqua* was fractionated on a solubility basis. The galactomannan was stirred in distilled water for 12 h at 10°C; the solution was removed by centrifugation and freeze-dried to yield the cold water soluble fraction. The residue was stirred in hot distilled water (90°C) for 2 h. The solution was again removed by centrifugation, and freeze-dried to yield the hot water soluble fraction. Equal proportions of the two fractions were obtained. Analysis indicated that the hot water soluble fraction and the cold water soluble fraction had galactose contents of 20% and 25%, respectively, compared with 23% for the whole galactomannan.

(iii) *α-Galactosidase treated galactomannans*

Coffee bean α-galactosidase was prepared following the method of Courtois & Petek (1966). The preparation contained a small but signifi-

TABLE 2

Galactomannan used	*Galactose content (%)*	*Minimum concentration of galactomannan (% w/v) which will gel*		*Plateau in net positive contribution to optical rotation when mixed with 0·05% agarose measured at 589 nm*
		0·05% Agarose	*0·025% Agarose*	
Ceratonia siliqua	23	0·05	0·6	0·028
Caesalpinia spinosa	25	0·1	1·5	0·017
Gleditsia triacanthos (sample 1)	24	0·15	2·0	0·016
Caesalpinia pulcherima	24	0·3	2·0	0·014
Gleditsia triacanthos (sample 2)	27	0·3	2·0	0·012
Gleditsia amorphoides	24	0·3	2·0	0·014
Cyamopsis tetragonolobus	39	0·7	3·0	0·008
Trigonella foenum-graecum	48	1·0	4·0	0·007

cant contamination of β-mannanase. *Cyamopsis tetragonolobus* galactomannan was incubated with this preparation at room temperature in citrate buffer (pH 4.6, 0.05 M). Aliquots were removed after 24, 48 and 96 h. In each case the enzymic reaction was stopped by boiling, and the modified galactomannan isolated, after dialysis, by freeze-drying. The three modified galactomannans had galactose contents of 25%, 20% and 14%, respectively. Because of the β-mannanase contamination, all the products were lower in chain length than the starting materials, and viscosity measurements indicated that molecular weight decreased with decrease in galactose content.

After the enzyme preparation had been stored in a refrigerator at 3°C for several months it was re-assayed for enzyme activities. Although β-mannanase activity was still present, it had significantly fallen. *Caesalpinia spinosa* galactomannan was modified with the aged enzyme preparation to give a product with galactose content 16%. Because of the lower level of β-mannanase contamination in this case, this particular product was less severely degraded in molecular weight.

(iv) *Glucomannans*

The glucomannan from *Amorphophallus konjac* was purified from milled dried tubers. This involved hot water (80°C) extraction followed by millipore filtration (1·2 μm) and freeze-drying. The milled dried tubers (Konnyaku Powder) were purchased from Saihara and Co. Ltd (Osaka, Japan). The glucomannan is partially acetylated, containing one acetate group per six hexose residues. It has been reported to contain glucose and mannose in the ratio 1:1·6 in a mixed β-1,4-linked linear molecule (Sugiyama, 1972). Spruce glucomannan was a gift from Professor G. O. Aspinall. It contains a negligible level of single D-galactose side chains, and its water solubility presumably results from a low molecular weight.

(v) *Substituted celluloses*

The amyloid from *Tamarindus indica* was purchased from T. M. Duche and Sons, Ltd (London, UK). It is based on a β-1,4-D-glucan main chain in which *c.* 75% of the glucose residues are substituted by monosaccharide or disaccharide side chains (Kooiman, 1961). The amyloid from *Annona muricata* was a gift from Dr P. Kooiman. Approximately 25% of the glucose residues in the main chain are substituted by side chains (Kooiman, 1967). Two samples of sodium carboxymethyl cellulose were used. A sample with degree of substitution 0·7 was purchased from British Celanese (code P8), while a sample with degree of substitu-

tion 0·4 was purchased from Hercules Inc. (Delaware, USA) (code 4M6F).

(vi) *Mixed linkage β-glucans*
The mixed linkage (β-1, 3/β-1,4) β-glucan from barley was a gift from Dr G. Bathgate.

(vii) *Xylans*
Sapote gum from *Achras sapota* was purchased from Stein Hall Ltd (New York, USA). Almost all the xylose residues in the main chain are substituted with side chains (Stephen & Schelpe, 1964). The corm sac polysaccharide from *Watsonia pyrimidata* was a gift from Professor A. M. Stephen. All the xylose residues in the main chain are substituted with side chains and some are substituted twice (Shaw & Stephen, 1966). The arbinoxylan from esparto grass was a gift from Professor G. O. Aspinall. It has a xylose to arabinose ratio of 6:1, and its water solubility presumably results from a low molecular weight.

(c) Analysis of galactomannans

Galactose and mannose contents of galactomannans were determined by Dr A. Morrison, by gas chromatography of the mixture of galactitol and mannitol hexa-acetates that resulted from hydrolysis, reduction and acetylation.

(d) Optical rotation measurements

Optical rotation measurements were carried out with the Perkin Elmer 141 polarimeter using 100 mm cells. Measurements were made at wavelengths of 365, 436, 546, 578 and 589 mm. The experimental procedures and precautions have been described earlier (Dea *et al.*, 1972).

(e) Measurement of rigidity modulus of gels

Rigidity modulus of gels during setting was measured by Mrs J. Boyd, using a modification of a Weissenberg Rheogoniometer. Gels of agarose/agar alone and mixed gels were made by heating to 95°C. In order to standardise the start time of the experiments, solutions were cooled quickly to 40°C and held at this temperature for 15 min. The samples were then cooled to 25°C where they were maintained throughout the course of the experiment. This enabled setting rates and final strength of the gels to be measured.

(f) Determination of gel points

The setting and melting temperatures of gels were determined using the sedimentation of glass beads by the procedures described previously (Dea *et al.*, 1972).

RESULTS

(a) Effect of galactomannan structure on their interaction with agarose

We have reported previously (Dea *et al.*, 1972) that the addition of *Ceratonia siliqua* galactomannan to non-gelling 0·05% agarose results in gelation, and changes the form of the optical rotation trace; the cooling curve is now a composite of the usual negative contribution from the agarose coil-to-helix conversion and a new positive contribution from a galactomannan transition which does not occur in the absence of agarose. The hysteresis now shows a complex butterfly form instead of the usual loop, which was interpreted as showing that, although the disorder–order conversions for the two polysaccharides occur together on the cooling curve, on re-heating, the order–disorder transition for the galactomannan can be caused to occur first (Fig. 1).

Gelling and non-gelling concentrations of agarose show identical optical rotation behaviour on cooling and reheating (Dea *et al.*, 1972). This indicates that the forms of the optical rotation changes on gelling and melting agarose are not artefacts of gel formation and gel strain, but originate from molecular effects. Segmented agarose, the product of the kink-splitting series of reactions (Dea *et al.*, 1972), has lost the ability to gel. On cooling, its solutions become cloudy and precipitation occurs. It is inferred that, on cooling, segmented agarose undergoes a coil-to-double-helix transition. These double helices immediately aggregate and precipitate, preventing optical rotation measurements. However, it is estimated from the temperature at which opacity occurs that the formation of double helices in the segments occurs at least 5°C lower than for comparable concentrations of agarose. When segmented agarose is mixed with *Ceratonia siliqua*, galactomannan gelation can occur (Dea *et al.*, 1972). Thus a mixture of segmented agarose (0·2%) and *Ceratonia siliqua* galactomannan (1%) forms a weak gel on cooling which is too cloudy to allow examination by optical rotation. The setting temperature for this gel is 24°C. This compares with a value of 32°C for the mixed gel containing agarose (0·2%) and *Ceratonia siliqua* galactomannan (1%). The melting temperatures are 65°C and 85°C, respectively.

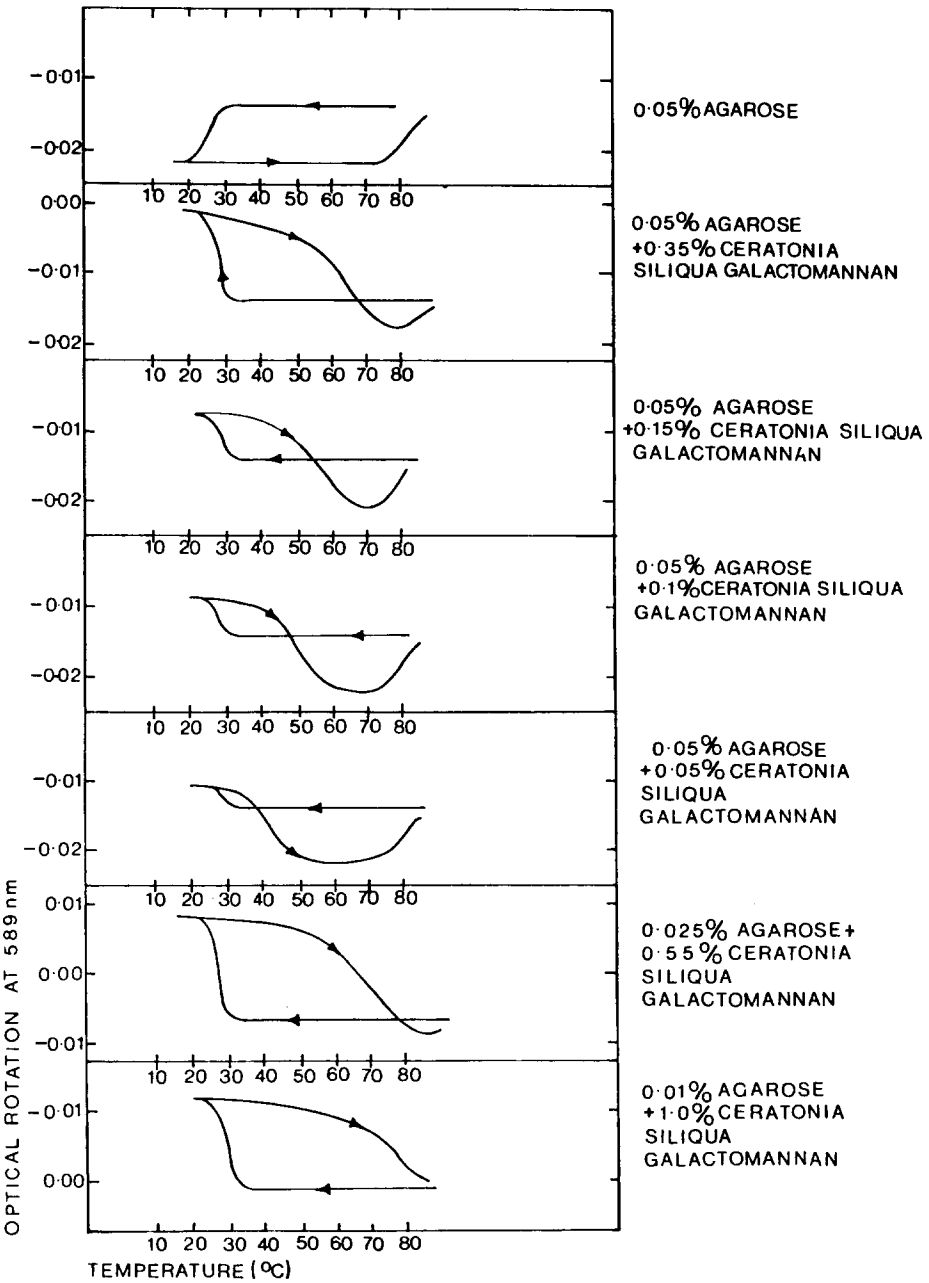

Fig. 1. Comparison of the optical rotation variations with temperature for agarose at a non-gelling concentration (0·05%), and a number of mixtures of agarose (0·05%, 0·025% and 0·01%) with *Ceratonia siliqua* galactomannan. Hot solutions were introduced into the polarimeter cell at 90°C, and these solutions were then cooled and reheated to give the hysteresis data.

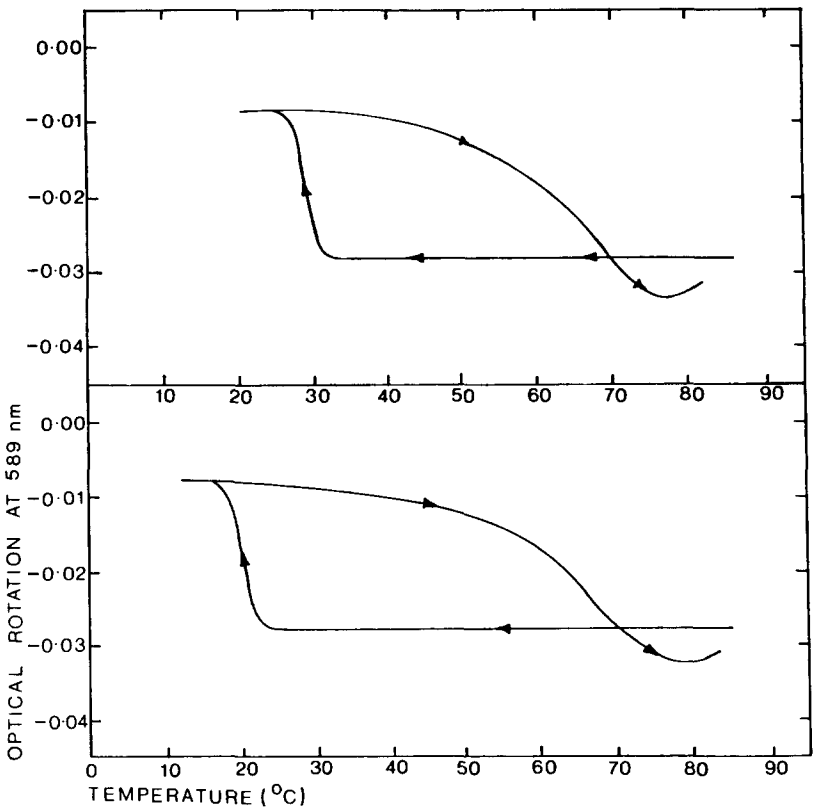

Fig. 2. Comparison of the optical rotation variations with temperature for (top) a gelling mixture of agarose (0·1%) and *Ceratonia siliqua* galactomannan (0·5%), and (bottom) a non-gelling mixture of segmented agarose (0·1%) and *Ceratonia siliqua* galactomannan (0·5%).

At lower concentrations of segmented agarose and the galactomannan, non-gelling mixtures are obtained. Figure 2 compares the optical rotation behaviour of a non-gelling mixture of segmented agarose (0·1%) and *Ceratonia siliqua* galactomannan (0·5%) with the gel obtained from the same levels of agarose and the galactomannan. The main difference is that the positive optical rotation transition occurs about 8°C higher in the case of the agarose mixture, in agreement with the conclusion that the double helices can form at higher temperatures for agarose than the segmented material. The fact that the non-gelling mixture shows the complex hysteresis behaviour is strong evidence that this optical rotation behaviour also originates from molecular effects.

The extent of interaction between agarose and galactomannans generally decreases with increase in galactose content (Dea *et al.*, 1972;

McCleary *et al.*, 1984). This is illustrated by examining the effect that addition of galactomannans to gelling concentrations of agarose can have on gel strength, by measuring the rigidity modulus with time as agarose and agarose/galactomannan mixed systems are gelled under constant temperature conditions. Figure 3 indicates the effect that 0·1% levels of three galactomannans, of widely differing galactose content, have on the gelling properties of 0·2% agarose. The small increase in gel strength shown by the highest galactose containing galactomannan (*Trigonella foenum-graecum*) is a real effect, and polysaccharides which show no co-gelling interaction with agarose (e.g. sodium alginate) give traces identical to agarose alone. Not only does the galactomannan with least galactose content result in the greatest increase in rigidity modulus, but it also causes the onset of gelation to start earlier. The addition of galactomannans to κ-carrageenan induces the formation of the carra-

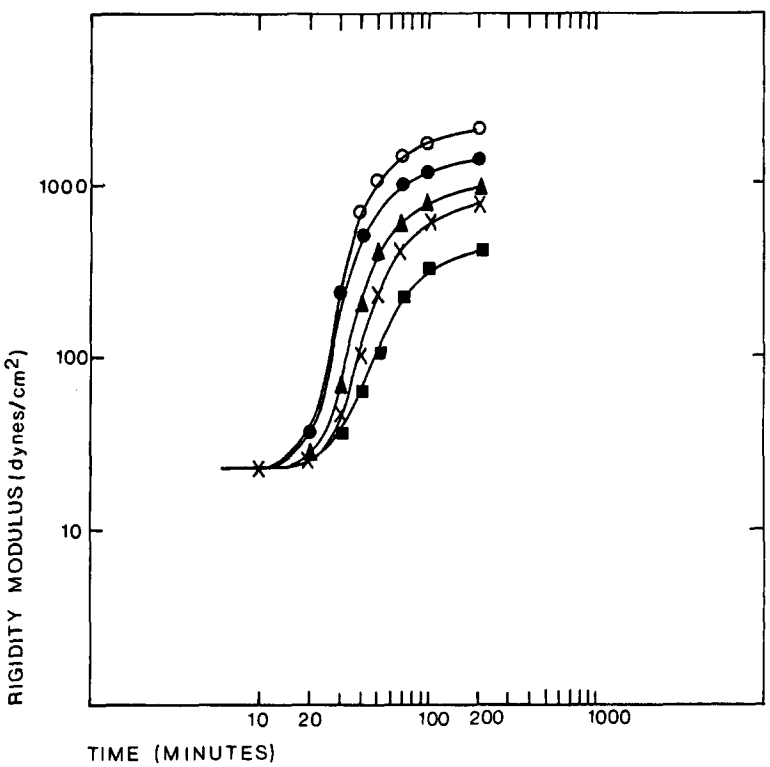

Fig. 3. Variation of rigidity modulus with time for the setting of 0·1% agarose (×), a mixture of 0·2% agarose and 0·1% *Ceratonia siliqua* galactomannan (○), a mixture of 0·2% agarose and 0·1% *Cyamopsis tetraganolobus* galactomannan (●), a mixture of 0·2% agarose and 0·1% *Trigonella foenum-graecum* galactomannan (▲), and a mixture of 0·2% agarose and 0·1% enzyme modified *Cyamopsis tetraganolobus* galactomannan (■).

geenan double helix and results in an increase in gel setting temperature (Dea *et al.*, 1972). Similar effects are observed for the interaction between galactomannans and xanthan (Dea *et al.*, 1977). However, there is no evidence that the addition of galactomannans to agarose increases the gelling temperature.

Evidence that the extent of interaction between agarose and galactomannans generally decreases with increase in galactose content is also illustrated by considering the minimum concentration of galactomannan required to cause non-gelling concentrations of agarose to gel (Table 2). This indicates that although there is a trend towards greater effectiveness at gelling low concentrations of agarose as the galactose content of the galactomannan decreases, the relationship is not straightforward. Thus, although the galactomannans from *Gleditsia triacanthos* (sample 1), *Caesalpinia pulcherima* and *Gleditsia amorphoides* have galactose contents (24%) less than that from *Caesalpinia spinosa* (25%), they are significantly less effective in co-gelling with agarose.

If, after correcting for the negative optical rotation of agarose, the magnitude of the galactomannan's positive contribution to optical rotation on cooling agarose/galactomannan mixtures to 20°C (i.e. below the gel point) is used as a measure of the extent of interaction between non-gelling agarose concentrations and *Ceratonia siliqua* galactomannan, we find that as galactomannan concentration increases the extent of interaction increases in absolute terms until the agarose is saturated (see Fig. 4). At the higher galactomannan concentrations the proportion of the interacting galactomannan decreases. All this would be expected if the binding obeyed any form of equilibrium law. Figure 1 shows the optical rotation hysteresis curves pertaining to the *Ceratonia siliqua* data in Fig. 4. The effect of systemic variation in the level of galactomannan addition to agarose (0.05%) on the optical rotation behaviour for the other galactomannans was obtained in the same way. The net positive contribution to optical rotation for mixtures of non-gelling agarose concentrations and the other galactomannans is also plotted in Fig. 4. In all cases the net positive contribution to optical rotation plateaus at higher galactomannan concentrations. The magnitude of this maximum in net positive contribution can be regarded as a measure of the ability of the galactomannan to interact with agarose, and the data are listed in Table 2. This again indicates that, although there is a trend towards greater interaction with agarose as the galactose content of the galactomannan decreases, the relationship is not straightforward. Recently, it has been shown that significant differences in the distribution of galactose groups along the mannan backbone (fine structure) exist for the galactomannans from *Ceratonia siliqua*, *Caesalpinia spinosa*, *Caesalpinia pulcherima* and *Gleditsia triacanthos*, and that this can influence their co-gelling proper-

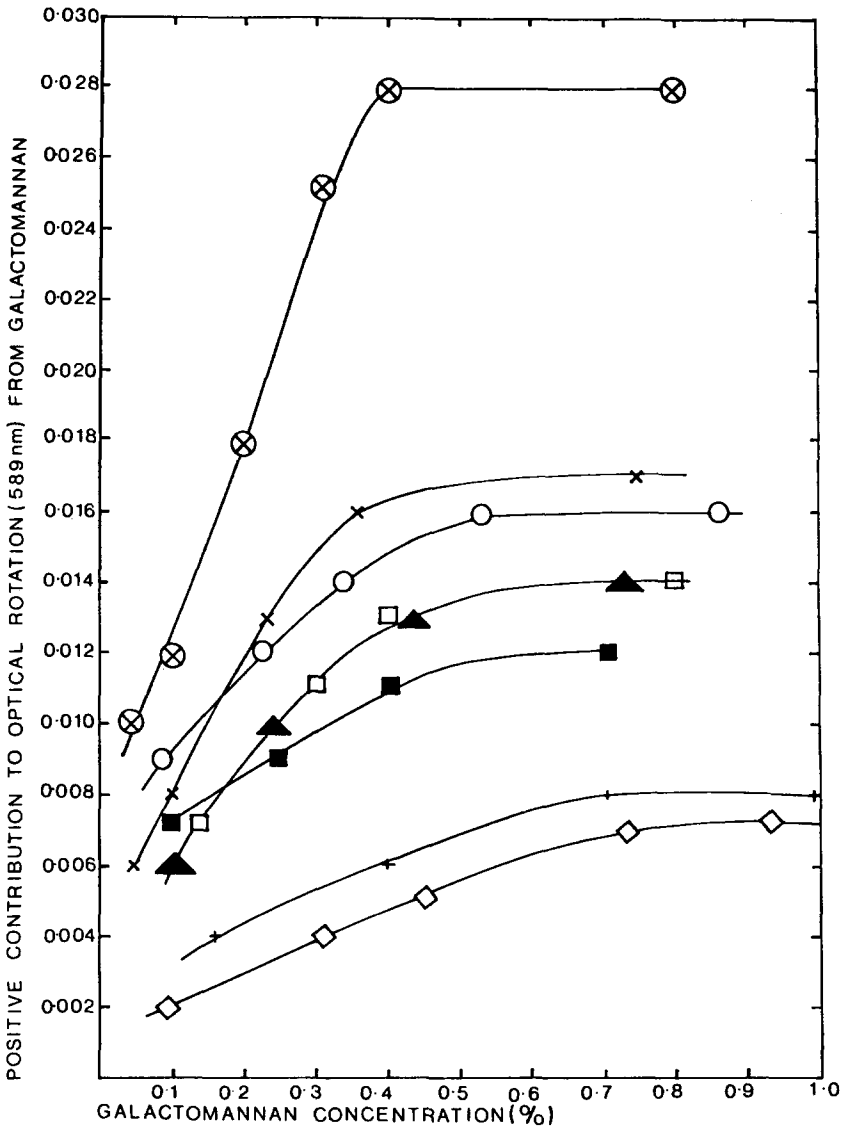

Fig. 4. Variation of the positive contribution to the optical rotation change, in mixtures of agarose (0·05%) and galactomannan, with galactomannan level. The galactomannans studied are those from *Ceratonia siliqua* (⊗), *Caesalpinia spinosa* (×), *Gleditsia triacanthos* sample 1 (○), *Gleditsia triacanthos* sample 2 (□), *Caesalpinia pulcherima* (▲), *Gleditsia amorphoides* (■), *Cyamopsis tetragonolobus* (+) and *Trigonella foenum-graecum* (◇).

ties (Dea *et al.*, 1986*a*, *b*). This phenomenon may be operating in these results.

From Fig. 1 it is clear that, for agarose mixtures with *Ceratonia siliqua* galactomannan, optical rotation changes between 20°C and 70°C on the

heating curve can be attributed to melting of the galactomannan ordered conformation. The midpoint of this transition increases by 20°C as the galactomannan concentration varies from 0·05% to 0·35% in the presence of 0·05% agarose. This trend is further illustrated by the optical rotation results from mixtures of 0·025% agarose with 0·5% *Ceratonia siliqua* galactomannan, and 0·01% agarose with 1·0% *Ceratonia siliqua* galactomannan, both of which gel at room temperature. Thus for the former the butterfly hysteresis curve is extremely exaggerated, and for the latter the negative loop has been completely replaced by a positive loop (Fig. 1). By increasing the ratio of the galactomannan to agarose, we are evidently increasing the concentration of binding sites for the agarose helices, so driving the melting transition to a higher temperature by a mass action phenomenon. This increase in hysteresis with galactomannan concentration is also accompanied by an increase in the gel melting point. Thus the gels from 0·05% agarose plus 0·35% galactomannan, and 0·05% agarose plus 0·15% galactomannan, melt at 71°C and 51°C, respectively.

We might suspect that superimposed on this effect could be an influence from microheterogeneity of the *Ceratonia siliqua* galactomannan molecules. They might be expected to carry a variety of binding sites with a spectrum of stabilities in the bound ordered state with agarose. Higher galactomannan concentrations would then make available not only a higher population of total binding sites but also a higher population of sites with higher affinity for agarose. Some indication for the importance of galactomannan fine structure in the interaction with agarose, and for the relative importance of microheterogeneity and mass action effects in controlling the hysteresis phenomena, can be obtained by comparing the effect of the different galactomannans on the optical rotation properties of mixtures with agarose. Figure 5 compares the optical rotation trace for agarose (0·05%) plus *Ceratonia siliqua* galactomannan (0·35%) with traces obtained for the galactomannans from *Caesalpinia spinosa*, *Caesalpinia pulcherima*, *Gleditsia triacanthos* (sample 1), *Gleditsia triacanthos* (sample 2) and *Gleditsia amorphoides* mixed at the same levels with agarose. Also shown in Fig. 5 is the optical rotation trace for agarose (0·05%) plus *Cyamopsis tetragonolobus* galactomannan (1·0%). The optical rotation behaviour of *Trigonella foenum-graecum* galactomannan is closely similar to the galactomannan from *Cyamopsis tetragonolobus* and is not shown.

There is a trend from a large hysteresis for the positive rise in optical rotation in the case of *Ceratonia siliqua* galactomannan, to little or no hysteresis in the case of *Cyamopsis tetragonolobus* galactomannan. Also, although the galactose contents of the first six galactomannans are very similar, there are large differences in hysteresis behaviour, which

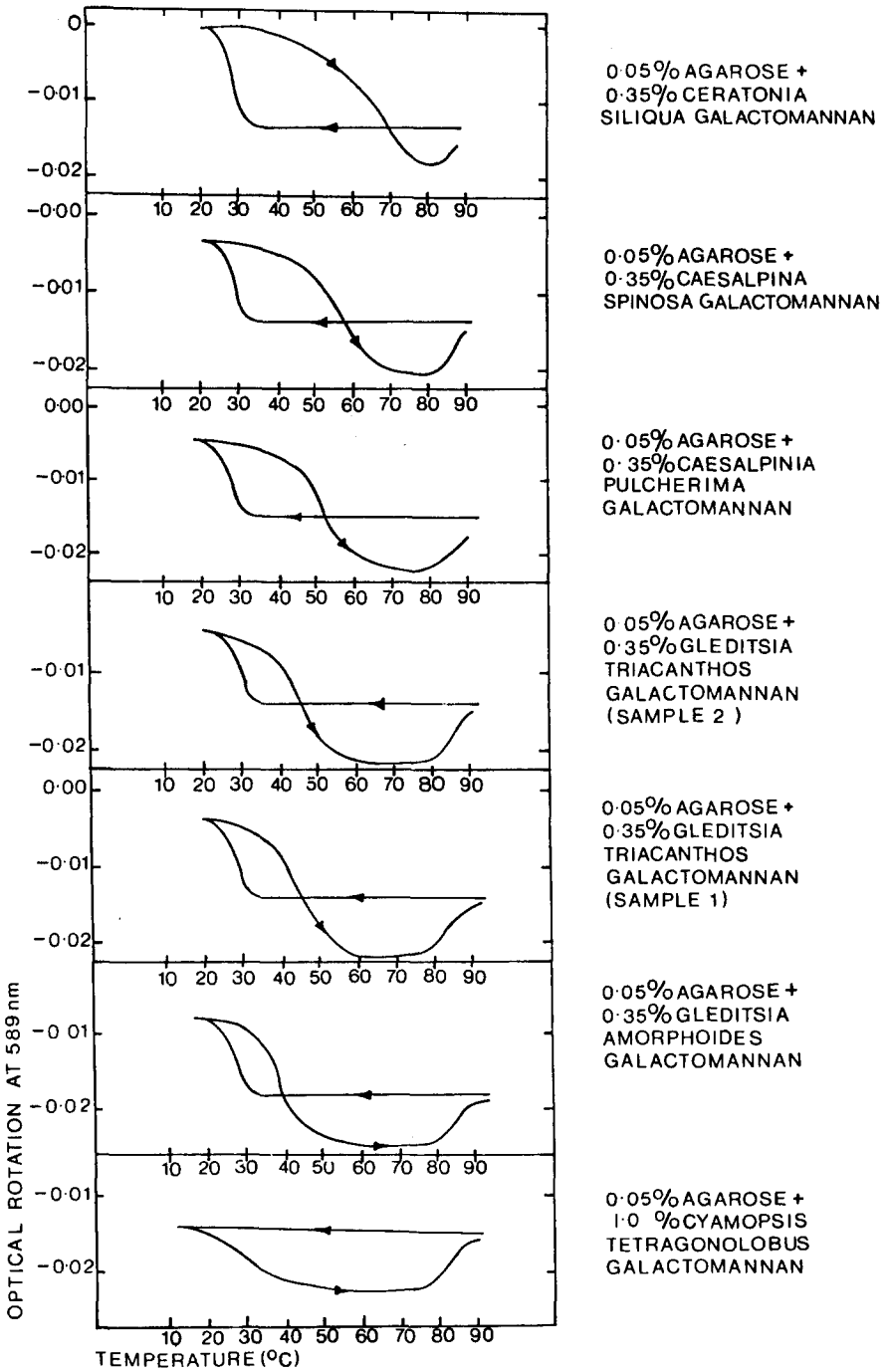

Fig. 5. Comparison of the optical rotation variations with temperature for a range of agarose–galactomannan mixtures.

strongly indicate significant differences in the fine structures of these galactomannans. In particular, the galactomannans from *Caesalpinia pulcherima*, *Gleditsia triacanthos* (sample 1) and *Gleditsia amorphoides*, which contain 24% galactose, show differing hysteresis for the positive rise in optical rotation, which are all smaller than the hysteresis behaviour shown by *Caesalpinia spinosa* galactomannan (25% galactose). Moreover, variation of agarose to galactomannan ratios indicates similar hysteresis behaviour as shown by *Ceratonia siliqua* galactomannan, but to varying extents. Thus the variation in hysteresis behaviour of agarose/-*Caesalpinia spinosa* galactomannan mixtures with galactomannan concentration is less than that of agarose/*Ceratonia siliqua* galactomannan mixtures, while the hysteresis shown by agarose mixed with the three *Gleditsia* galactomannan systems varies only slightly with galactomannan concentration. This large difference in the sensitivity of hysteresis to galactomannan concentration suggests that mass action plays a relatively minor role. Rather, this hysteresis effect is a probe for the width of the molecular spectrum of galactomannan, since at low ratios of agarose to galactomannan the agarose binds that fraction of galactomannan with which it interacts best. Thus *Ceratonia siliqua* galactomannan has a wide spectrum varying from weakly binding to quite strongly binding components, while the *Gleditsia* galactomannans have a rather narrow spectrum of weakly binding molecules. This is further emphasised by the fact that cold water extract of *Gleditsia triacanthos* whole seeds interacts with agarose to much the same extent as the hot extract of these whole seeds (see Fig. 5 and Table 2).

The suggestion that *Ceratonia siliqua* galactomannan has a wide molecular spectrum is supported by the fact that it can be fractionated on the basis of differential water solubility (Hui & Neukom, 1964), and freeze–thaw treatment of mixed agarose/galactomannan gels (Dea *et al.*, 1972). In both cases the fractionation is on the basis of galactose content. Our standard *Ceratonia siliqua* galactomannan (23% galactose) was fractionated by differential water solubility to give a cold water soluble fraction (25% galactose) and a hot water soluble fraction (20% galactose). Mixtures of the whole galactomannan and the two fractions (0·35%) with agarose (0·05%) were examined by optical rotation, and the traces are shown in Fig. 6. It can be seen that the extent of interaction between agarose and galactomannan, as measured by both the positive rise in optical rotation and the width of the hysteresis curve, increases with decrease in galactose content.

It has previously been reported that *Cyamopsis tetragonolobus* galactomannan can be treated with a purified preparation of α-D-galactosidase from lucerne seeds to give products depleted in galactose

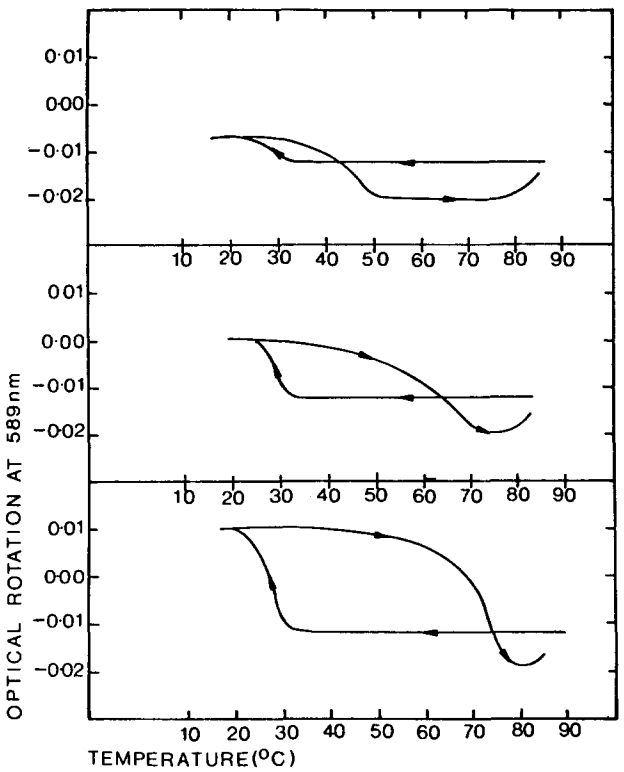

Fig. 6. Comparison of the optical rotation variations with temperature for (top) a mixture of agarose (0·05%) and the cold water soluble fraction of *Ceratonia siliqua* galactomannan (0·35%), (middle) a mixture of agarose (0·05%) and *Ceratonia siliqua* whole galactomannan (0·35%), and (bottom) a mixture of agarose (0·05%) and the hot water soluble fraction of *Ceratonia siliqua* galactomannan (0·35%).

without any reduction in polysaccharide chain length, which show improved gelling interactions with xanthan and agarose (McCleary *et al.*, 1984). In this study we have modified *Caesalpinia spinosa* galactomannan with a partially purified α-D-galactosidase preparation from coffee beans to reduce the galactose content from 25% to 16%. Figure 7 shows the optical rotation behaviour of the product mixed with agarose compared with that for the native galactomannan. It can be seen that the modified product interacts more with agarose than the native material, as shown by the greater positive contribution to optical rotation, and that its binding is stronger, as shown by the wider hysteresis. However, using both these criteria the modified galactomannan interacts no more strongly with agarose than does the hot water soluble fraction of

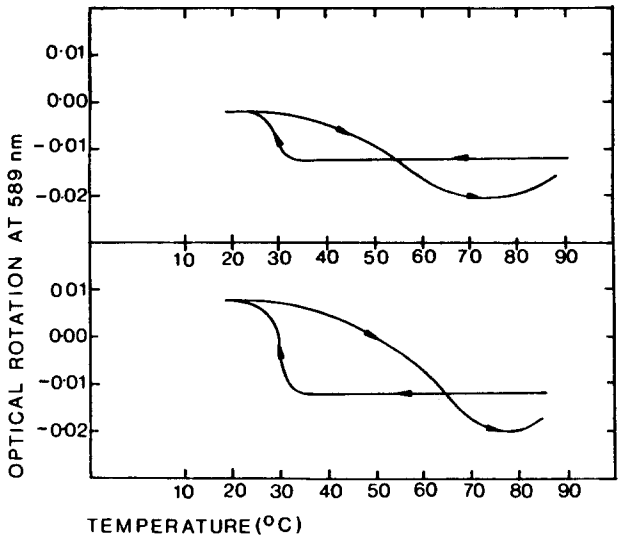

Fig. 7. Comparison of the optical rotation variations with temperature for (top) a mixture of agarose (0·05%) and *Caesalpinia spinosa* galactomannan (0·35%), and (bottom) a mixture of agarose (0·05%) and enzyme treated *Caesalpinia spinosa* galactomannan (0·35%).

Ceratonia siliqua galactomannan (Fig. 6), although it has a much lower galactose content (16% compared with 20%). This is a further indication of the importance of fine structure in determining the strength of interactions with agarose. Despite this increased ability to interact with the agarose double helix on a molecular level, the modified galactomannan shows no greater effect on gelling interactions with agarose than the native *Caesalpinia spinosa* galactomannan. This is presumably because the modified product is of lower chain length due to contamination of the α-D-galactosidase preparation by β-D-mannanase.

So far we have considered the role of the level and distribution of galactose substituents in the galactomannans on their interaction with agarose. Examination of the galactomannan from *Crotalaria mucronata* demonstrates the importance of the main chain structure, since this galactomannan has 22% of the linkages in the main chain not β-1,4-linked (Unrau & Choy, 1970*b*). Despite the fact that this galactomannan has a fairly low galactose content (27%), it showed no evidence from gelling studies or optical rotation studies of interacting with agarose. This indicates the importance of β-1,4-linked regions in the D-mannan backbone for the interaction with agarose.

(b) Effect of substitution along the agarose molecule on the interaction with galactomannans

In the carrageenan series, κ-carrageenan, which bears one sulphate group per disaccharide repeating unit, exhibits co-gelling interactions with the lightly substituted galactomannans from *Ceratonia siliqua* and *Caesalpinia spinosa*, while ι-carrageenan, which bears two sulphate groups per disaccharide repeating unit, does not interact. A less sulphated κ-carrageenan variety, furcellaran, exists which contains a sulphate group for every two disaccharide repeating units. Furcellaran shows a greater capacity to interact with galactomannans than κ-carrageenan. This is illustrated by its interaction with the heavily substituted galactomannan from *Cyamopsis tetragonolobus*. This galactomannan gels a non-gelling concentration (0·3%) of furcellaran when added at a 3% level. In contrast, no co-gelling interaction can be demonstrated between κ-carrageenan and *Cyamopsis tetragonolobus* galactomannan (Dea *et al.*, 1972).

A plausible reason why agarose interacts very much better with galactomannans than the carrageenans is therefore because it is completely non-substituted. It is thus of interest to examine the co-gelling interaction properties with galactomannans of a range of naturally substituted gel-forming agars. The most common natural substituents in the agar series are *O*-methyl groups (Araki, 1966). Comparison of non-substituted agarose with a moderately *O*-methylated agar (sample 8 in Table 1 with a OCH_3 content of 2·26%, equivalent to 12·8% 6-*O*-methyl-D-galactose) indicates that the presence of *O*-methyl groups hinders the co-gelling interaction. Both the agarose and the agar form gels at a concentration of 0·1%. However, while 0·05% agarose is gelled by addition of only 0·05% *Ceratonia siliqua* galactomannan (Table 2), the addition of 0·6% galactomannan is required to gel 0·05% of the *O*-methylated agar. The poorer co-gelling interaction of the *O*-methylated agar compared with agarose is also demonstrated by measuring the gel strengthening effect of adding galactomannans to gelling concentrations of these materials. Addition of 0·1% *Ceratonia siliqua* galactomannan to 0·2% agarose resulted in a 50% increase in rigidity modulus (see Fig. 3). In contrast, addition of 0·1% galactomannan to 0·2% of the *O*-methylated agar resulted in a 15% increase in rigidity modulus.

Further evidence of the poorer co-gelling interaction of this *O*-methylated agar is obtained from freeze–thaw experiments on mixed gels. Gels were prepared comprising 0·5% agarose or the agar and 0·1% *Ceratonia siliqua* galactomannan. On freeze–thaw treatment, each gel shrank and released about half the volume as fluid, which was analysed

for galactomannan. The analyses indicated that 80% of the galactomannan was bound to the agarose network, compared with only 55% bound to the *O*-methylated agar network.

The presence of *O*-methylation has a large effect on the temperature dependence of optical rotation for agars. Non-substituted agarose shows a very sharp cooling and reheating optical rotation transition with a wide hysteresis (55°C wide). *O*-Methylated agars give similar behaviour but have less sharp transitions, higher gelling temperatures (cooling transition) and a range of melting temperatures (re-heating transitions) (Arnott *et al.*, 1974*a*). Figure 8 compares the temperature dependence of optical rotation for 0·1% concentration of non-substituted agarose and the *O*-methylated agar studied above, together with the effect that the addition of 0·3% *Ceratonia siliqua* galactomannan has on the optical rotation behaviour. The lower positive contribution to optical rotation from the galactomannan in the case of the agar is further evidence that the presence of *O*-methyl groups hinders the interaction.

The positive contribution to the change in specific rotation of *Ceratonia siliqua* galactomannan in the presence of agar can be taken as a good measure of the co-gelling interactivity of a range of agars with widely different levels of *O*-methylation. Measurements were carried out on mixed systems of agar (0·1%) and galactomannan (0·3%). Figure 9 shows how this measure of interactivity varies with *O*-methyl content. It can be seen that even *O*-methyl contents as low as 0·43% reduce the interaction. Agars which contain more than one *O*-methyl group for every two disaccharide repeating units (samples 14 and 15 in Table 1, alkali modified porphyran and *Rhodomela larix* agar) give no perceptible positive contribution to the change in specific rotation when mixed with the galactomannan at these levels. However, for such highly *O*-methylated agars, bulk measurements indicate that a co-gelling interaction still occurs, but that it is very weak.

The relationship shown in Fig. 9 is a relatively smooth pattern except for two agars with *O*-methyl contents of 1·91% and 2·37% (Table 1, samples 6 and 9, respectively), where the extent of interaction is less than would be expected. It has been shown previously (Guiseley, 1970) that a good relationship exists between gel setting temperature and *O*-methyl content of agars. This was determined by studying 48 agar samples. In the present study, 16 of Guiseley's original samples were examined, and the two samples which do not conform to the norm also did not conform to Guiseley's relationship. This would indicate that in some instances the position and/or distribution of *O*-methyl groups along the agar chain, rather than simply the *O*-methyl content, may be important in controlling the interaction with galactomannans.

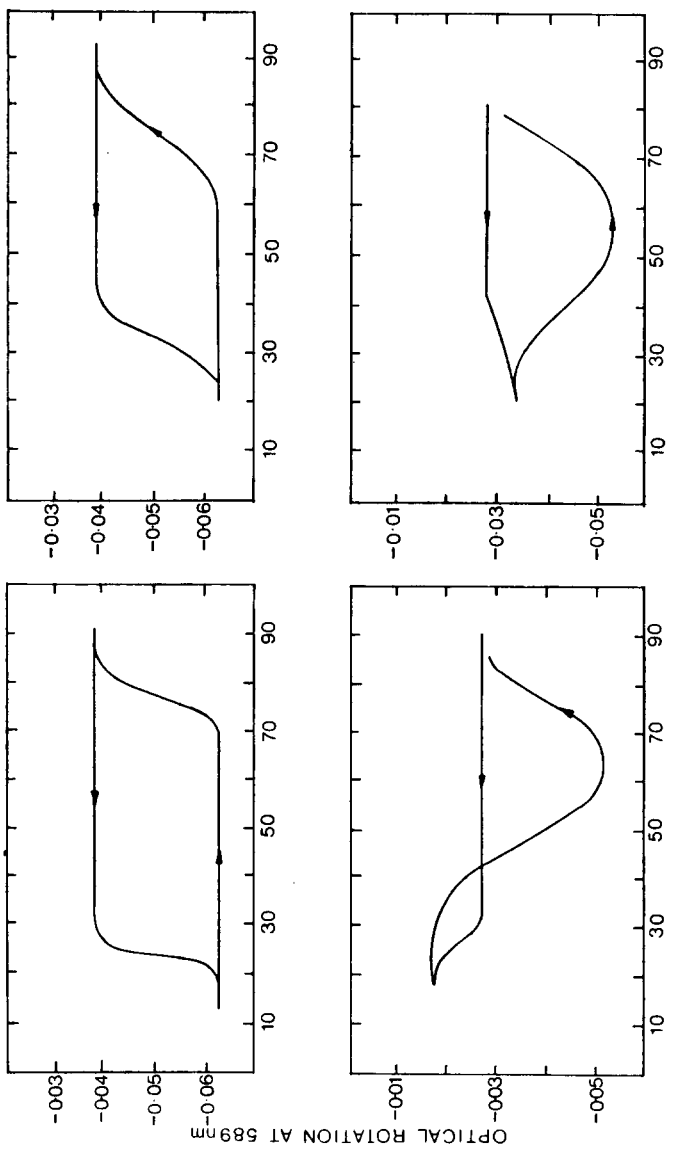

Fig. 8. Comparison of the optical rotation variations with temperature between (top) agar (0·1%) and (bottom) a mixture of agar (0·1%) and *Ceratonia siliqua* galactomannan (0·3%), for unsubstituted agarose (on the left) and a natural moderately *O*-methylated agar (on the right).

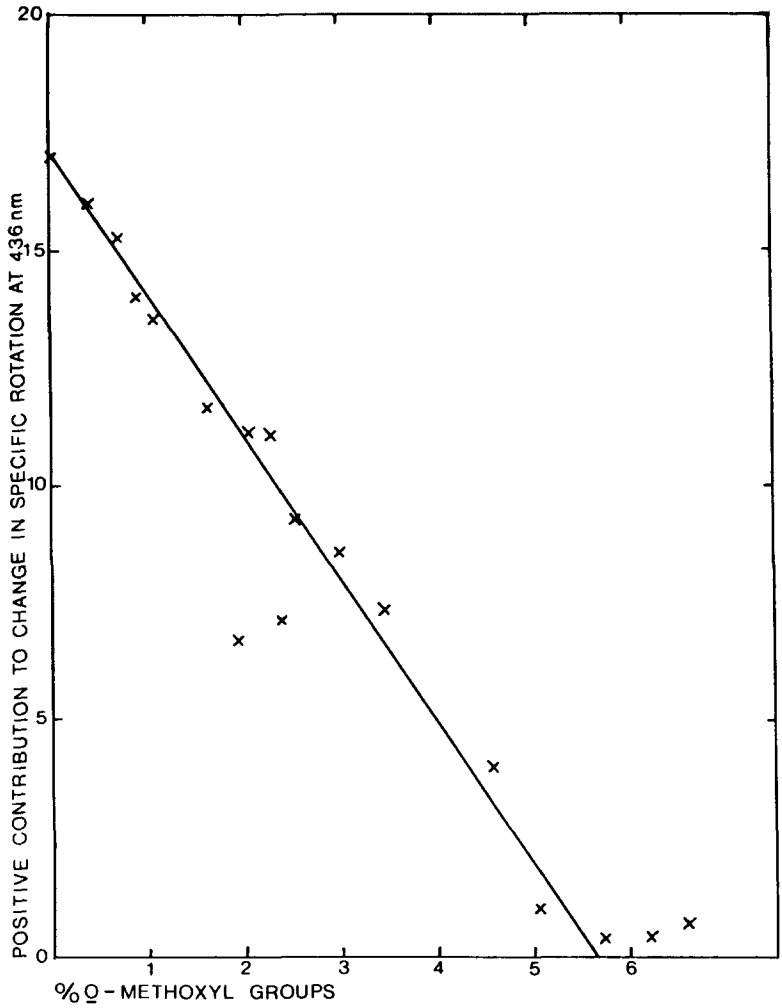

Fig. 9. Comparison of the positive contribution to the change in specific rotation on cooling mixtures of agar (0.1%) and *Ceratonia siliqua* galactomannan (0·3%), with variation in the *O*-methyl content of the agar.

Agars can also be naturally substituted by pyruvic acid (Hirase, 1957; Duckworth *et al.*, 1971). The agar from *Gracilaria compressa* has been reported (Young *et al.*, 1971) to contain a particularly high level of combined pyruvic acid (3%) which corresponds to the presence of D-galactose and 4,6-*O*-carboxyethylidene-D-galactose in a ratio of about 7:1. Presumably because this bulky substituent is negatively charged, this agar gives less aggregated/less turbid gels, and the minimum gelling concentration is much higher (0·7%) than for non-charged agars. *Garcilaria*

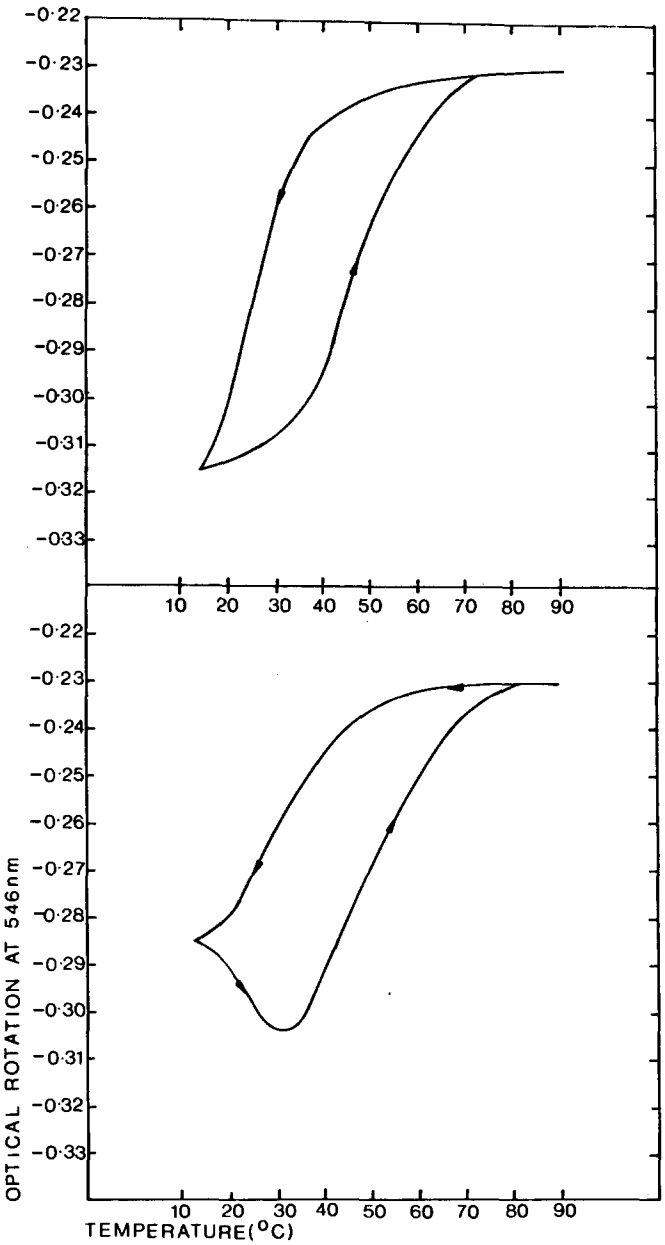

Fig. 10. Comparison of the optical rotation variations with temperatures for (top) a gelling concentration of *Gracilaria compressa* agar (0·7%), and (bottom) *Gracilaria compressa* agar (0·7%) and *Ceratonia siliqua* galactomannan (2·1%).

compressa agar exhibits co-gelling interactions with galactomannans, and on examining the interaction with *Ceratonia siliqua* galactomannan by optical rotation a positive contribution to the optical rotation from the galactomannan is observed (Fig. 10). When account is taken of the different concentrations used in this experiment (0·7% agar, 2·1% galactomannan), the positive contribution to optical rotation from the galactomannan is less than that obtained with an agar *O*-methylated to the same extent. This is what would be expected on steric grounds, since carboxyethylidene groups are much more bulky than *O*-methyl substituents.

A few native gel forming agars have been shown to be highly *O*-sulphated (Hirase & Watanabe, 1973; Penman & Rees, 1973). In the agar from *Gloeopeltis cervicornis* all the D-galactose residues are 6-*O*-sulphated and 20% of the 3,6-anhydro-L-galactose residues are 2-*O*-sulphated (Penman & Rees, 1973). This degree of sulphation is similar to that found in κ-carrageenan. Because of this negatively charged substitution, this agar exhibits unusual gelling properties. Its minimum gelling concentration is 2%, and the gels formed are only slightly turbid, indicating a low degree of aggregation within the gel network. At 3% concentration the hysteresis behaviour of gel setting (20°C) and gel melting (32°C) is much less than for the other agars studied. This is also shown by the temperature dependence of optical rotation (Fig. 11).

Gloeopeltis cervicornis agar exhibits co-gelling interactions with galactomannan. Thus a non-gelling 1% concentration of the agar is gelled by addition of 1% *Ceratonia siliqua* galactomannan. Addition of this galactomannan to gelling concentrations of the agar results in an increase in gel strength, and raises the gel melting temperatures. Thus, while a 3% gel of the agar alone melts at 32°C, the mixed gel of 3% agar plus 1% galactomannan melts at 42°C. The presence of galactomannan has only a small effect on the temperature dependence of optical rotation for the agar. As in the case of the heavily *O*-methylated agars, there is no detectable positive contribution to optical rotation from the galactomannan for this system. There is, however, evidence that the presence of the galactomannan stabilises the ordered conformation of the agar, since the width of the hysteresis loop is increased from 10°C for the 3% agar alone to 14°C for the mixed 3% agar/1% galactomannan gel.

In the case of *Gloeopeltis* agar, once the cooling optical transition has commenced readings are not stable in the long term, but rather gradually change to the corresponding position on the equilibrium heating curve. Thus a reading on the cooling curve of the optical rotation trace for 3% *Gloeopeltis cerviconis* agar at 22°C (A in Fig. 11) gradually drops to the

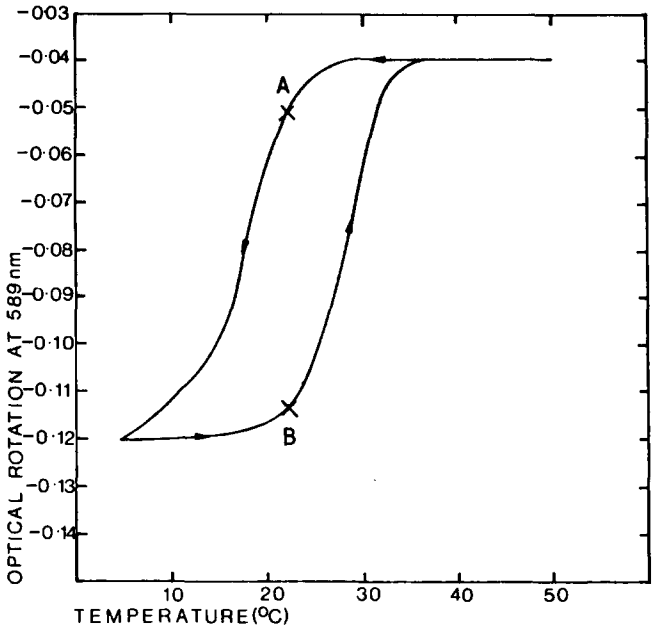

Fig. 11. Variation of optical rotation with temperature for a gelling concentration (3·0%) of *Gloeopeltis cervicornis* agar.

equilibrium heating curve (B in Fig. 11) over 7 days if the system is held at constant temperature. This slow transition occurs in half the time for a mixed system of 3% agar and 1% *Ceratonia siliqua* galactomannan, and is further evidence for an interaction between the two polysaccharides.

(c) Effect of structural variation in the main chain of 1,4-linked β-D-glycans on the interaction with agarose

1,4-Linked β-D-glucomannan, like 1,4-linked β-D-mannan, is insoluble in water. There are, however, substituted glucomannans found in nature which are water soluble. Thus the glucomannan food reserve polysaccharide from the tubers of *Amorphophallus konjac* is rendered soluble by a low degree of acetylation: approximately one out of every six glycosyl residues is acetylated (Sugiyama *et al.*, 1971). Evidently these occasional acetyl groups along the chain sterically prevent the aggregation of the D-glucomannan backbone, because deacetylation in dilute alkali leads to precipitation of pure glucomannan. It is not known whether there are regions of the backbone which are acetate rich and acetate free, or whether the acetates are relatively evenly distributed (Dea & Morrison, 1975). The structural evidence available suggests that

the mannose and glucose residues are distributed fairly evenly along the main chain, rather than in mannose-rich and glucose-rich blocks.

Since the backbone of this glucomannan differs from galactomannans only in the orientation of the hydroxyls on C2 of the glucose residues (38% of the main chain) (Kato & Matsuda, 1969), tests were carried out to determine whether it interacts with agarose. Its ability to gel non-gelling agarose (0·05%) ranked about equally with that of *Ceratonia siliqua* galactomannan. However, the temperature dependence of optical rotation for agarose/glucomannan mixtures differs significantly from that shown by agarose/*Ceratonia siliqua* galactomannan mixtures. Thus, while agarose/galactomannan mixtures show net positive optical rotation changes on cooling, agarose/glucomannan mixtures only show a reduced negative transition.

The equal co-gelling properties of *Amorphophallus konjac* glucomannan and *Ceratonia siliqua* galactomannan with agarose refer to both gel strength and gel melting and setting temperatures. *Amorphophallus konjac* glucomannan also exhibits a co-gelling interaction with κ-carrageenan and again this effect is equal to that shown by *Ceratonia siliqua* galactomannan. It is interesting to note, however, that the parity in co-gelling properties of these polysaccharides is not reflected in their interaction with xanthan (Dea *et al.*, 1977). In this case the glucomannan is much more effective than the galactomannan; much less is required to give gelling properties, and the melting points of the mixed gels are significantly higher.

The plant seed amyloids and the galactomannans have the same biological function (food store), and have obvious similarities in structure. In the amyloids, a cellulose main chain is solubilised by substitution with D-xylopyranosyl monosaccharide stubs and 2-*O*-β-D-galactopyranosyl-D-xylopyranosyl disaccharide stubs via α-D-1,6-linkages to certain of the D-glucosyl residues (Kooiman, 1967). The amyloids from both *Tamarindus indica* and *Annona muricata* were found to gel 0·05% agarose at levels of 0·9%. The interaction between these substituted 1,4-linked β-D-glucans and agarose is therefore quite weak, and is intermediate between that shown by the galactomannans from *Cyamopsis tetragonolobus* and *Trigonella foenum-graecum*. The interaction between agarose and these amyloids was also examined using temperature dependence of optical rotation. As in the case of the galactomannans, the interaction is evidenced by a positive contribution in optical rotation. The magnitude of this positive contribution is virtually identical for both amyloids, and only large enough to cancel the normal negative transition of agarose. This absence of a net positive optical rotation transition is a further indication of a strong similarity to the interaction of agarose with highly sub-

stituted galactomannans (Fig. 5). On reheating agarose/amyloid mixtures the optical rotation hysteresis behaviour is again very similar to that of agarose mixtures with the high galactose containing galactomannans, indicating that the amyloid–agarose associations which form on cooling dissociate very easily on reheating.

All the evidence therefore indicates that these two 1,4-linked β-D-glucan based amyloids interact with agarose, but only to the same extent as highly substituted galactomannans (40–48% galactose). The two amyloids, however, differ significantly in the degree of substitution along the main chain. For the amyloids from *Annona muricata* and *Tamarindus indica*, 25% and 75%, respectively, of the main chain glucose residues are substituted (Kooiman, 1961, 1967). This might suggest that the degree of substitution along the amyloid molecule has little effect on the extent of interaction with agarose, and would therefore constitute a major difference from galactomannans. However, viscosity measurements indicate that, for the samples used, the amyloid from *Annona muricata* was significantly lower in molecular size. In addition, nothing is known about the distribution of substituents along the main chain of amyloids, and such structural differences would be expected to materially effect interaction properties. A wider range of amyloids would therefore need to be examined before any conclusions could be made regarding the role of the extent of substitution in determining the degree of interaction with agarose.

Further evidence that 1,4-linked β-D-glucans can interact with agarose was obtained by examining mixtures of carboxymethyl-celluloses with agarose. Two commercially available carboxymethyl-celluloses with different extents of substitution along the main chain (40% and 70%) were investigated. The same amount of each (0·7%) was required to gel 0·05% agarose. The interaction between agarose and these two carboxymethyl-celluloses was also examined using temperature dependence of optical rotation. The results for each were identical, and closely similar to those obtained with the amyloids. In addition, they were also compared for the ability to increase the gel strength of 0·2% agarose when added at a 0·1% level. The measured rigidity moduli for the mixed gels were the same for both carboxymethyl-celluloses, and showed the same increase over pure agarose as the heavily substituted galactomannan from *Trigonella foenum-graecum* (Fig. 3).

β-D-Glucans show the same overriding importance of β-1,4-linkages for the interaction with agarose as the β-D-mannans. This was demonstrated by examining a barley β-glucan, with approximately the same number of β-1,4-linkages and β-1,3-linkages in the main chain. This material showed no evidence from gelling studies or optical rotation studies of interacting with agarose.

The interaction of agarose with two heavily substituted 1,4-linked β-D-xylans was also studied. Using the same gelation and optical rotation criteria, the heavily substituted xylans from sapote gum (Stephen & Schelpe, 1964) and *Watsonia pyrimidata* corm sacs (Shaw & Stephen, 1966) were found to interact, albeit weakly, with agarose. It is significant that these heavily substituted xylans interact with agarose; almost all the xylose residues in sapote gum are substituted, while some of the xylose residues in the *Watsonia* polysaccharide are even disubstituted. Solution studies (Dea *et al.*, 1973) with a self-associating hemicellulose xylan system have indicated that the arabinofuranosyl side chains have an unusual function, in that they do not cause termination of binding sites. Rather the associations remain, but in a modified form. A similar role for side chains in the association of *Watsonia* polysaccharide and sapote gum with agarose would explain why these heavily substituted D-xylans interact as well as they do with agarose. This demonstration of an interaction between the *Watsonia* polysaccharide and agarose is in agreement with observations made during chromatographic studies of the xylan (S. C. Churms & A. M. Stephen, pers. comm.). Chromatography on polyacrylamide gel resulted in a recovery of *Watsonia* polysaccharide in excess of 90%. In marked contrast, chromatography on agarose beads gave a recovery of only 30%.

(d) Effect of molecular size of 1,4-linked D-glycans on the interaction with agarose

Earlier, the interaction properties of α-galactosidase modified *Caesalpinia spinosa* galactomannan were discussed. This galactomannan had a reduced galactose content (16% compared with 25%), and using optical rotation criteria interacted significantly more on a molecular level with agarose than the native *Caesalpinia spinosa* galactomannan. However, its gelling interactions with agarose are no greater than the native material. This is ascribed to a decrease in polysaccharide chain length brought about by contaminating β-mannase activity in the α-galactosidase preparation. Mixed agarose–galactomannan gels are more turbid than agarose gelled alone, and this turbidity increases with decrease in galactose content for native galactomannans. Mixed gels of agarose with enzyme modified *Caesalpinia spinosa* galactomannan were significantly more turbid than the corresponding gel made with native *Caesalpinia spinosa* galactomannan.

Another enzyme preparation more severely contaminated with β-mannanase was used to carry out a series of modifications on *Cyamopsis tetragonolobus* galactomannan. Using increasing incubation times, three enzyme-modified samples were prepared, having galactose contents of

25%, 20% and 14%. Because of the β-mannanase contamination, the decrease in galactose content was accompanied by a decrease in polysaccharide chain length, as indicated by the drop in viscosity of the reaction mixtures.

The enzyme-modified *Cyamopsis tetragonolobus* galactomannan with 25% galactose content was less effective at gelling 0·05% agarose than the native galactomannan. In both its ability to gel 0·05% agarose and to increase the rigidity modulus of 0·2% agarose it showed the same effectiveness as the highly substituted *Trigonella foenum-graecum* galactomannan (Fig. 3). Despite this poor gelling activity, optical rotation studies (both positive contribution to optical rotation and width of hysteresis) indicated that it interacted more with agarose on a molecular level than the native galactomannan.

The low molecular weight enzyme-modified *Cyamopsis tetragonolobus* galactomannan containing 20% galactose did not gel 0·05% agarose. Instead it caused rapid precipitation from cooled mixed solutions. Furthermore, when 0·1% of the modified galactomannan was mixed with 0·2% agarose an extremely turbid gel was obtained which had a lower rigidity modulus than 0·2% agarose alone (see Fig. 3). A mixture of 0·2% agarose and 0·5% modified galactomannan when cooled gave a severely syneresing gel, and a collapsed gelatinised precipitate was obtained when 1% modified galactomannan was added to 0·2% agarose.

The lowest molecular weight, lowest galactose containing (14%) enzyme-modified *Cyamopsis tetragonolobus* galactomannan was even more effective at preventing agarose forming gels. Thus both 0·2% agarose with 0·1% modified galactomannan and 1·5% with 0·5% modified galactomannan gave gelatinous precipitates. This particular modified galactomannan was also examined by mixing with gelling concentrations of κ-carrageenan and ι-carrageenan. The addition of 1% modified galactomannan to 3% κ-carrageenan gave a more turbid weaker gel than 3% κ-carrageenan alone, and the addition of 2% modified galactomannan resulted in a gelatinous precipitate. With ι-carrageenan, however, no gel weakening activity was obtained, even when 5% modified galactomannan was added to 3% ι-carrageenan. The low molecular weight galactomannan therefore has gel weakening properties on mixing with agarose and κ-carrageenan, but not with ι-carrageenan. This same trend is observed for the co-gelling activity of native galactomannans, indicating that the underlying mechanisms are the same.

The prime importance of low polysaccharide chain length for this gel weakening activity of galactomannans was shown by examining the interaction properties of an alkali depolymerised sample of *Cyamopsis tetragonolobus* galactomannan. Although severely reduced in molecular

weight, this galactomannan had the same galactose content as the native material. At a level of 0·5% it precipitated 1% agarose, and gave a weaker and very turbid 1·5% agarose gel. This low molecular weight galactomannan also precipitated 3% κ-carrageenan when added at a level of 2%, but had no effect on the gelling properties of ι-carrageenan. Further evidence for the importance of low polysaccharide chain length was shown by examining the properties of samples of spruce galactoglucomannan and esparto grass arabinoxylan. Both these polysaccharides are low in molecular weight. On cooling 0·05% agarose with 1% of the spruce polysaccharide, a rapid precipitation occurred rather than gelation. However, a mixture of agarose (0·2%) and spruce polysaccharide (0·1%) gave a more turbid gel with a slightly higher rigidity modulus than 0·2% agarose alone. The increase in rigidity modulus obtained was similar to that obtained with the highly substituted galactomannan from *Trigonella foenum-graecum*. This would suggest that while the chain length of the galactoglucomannan is too small to permit the cross-linking and gelling of non-gelling agarose concentrations, it is still long enough to cross-link and strengthen agarose gels. As in the case of the spruce polysaccharide, cooling a non-gelling concentration of agarose (0·05%) with the arabinoxylan (1%) gave rapid precipitation rather than gelation. In contrast to the spruce polysaccharide, however, addition of 0·1% arabinoxylan to 0·2% agarose gave a more turbid gel with a lower rigidity modulus than 0·2% agarose alone, and 0·2% agarose was prevented from gelling by the addition of 1% arabinoxylan.

These observations strongly indicate that the co-gelling activity of high molecular weight 1,4-linked β-D-glycans and the gel weakening activity of low molecular weight 1,4-linked β-D-glycans have the same molecular basis. In the case of co-gelling activity, two factors can be considered as important. First, molecular association of regions of the 1,4-linked β-D-glycan chain to aggregated double helices of agarose or κ-carrageenan will lead to cross-linking of double helical aggregates. In non-gelling concentrations of agarose or κ-carrageenan this leads to complete gel network formation and for gelling concentrations of agarose and κ-carrageenan the strength of the gel network is increased. Secondly, the interaction leads to an increase in the extent of aggregation of the double helices, as indicated by the increase in turbidity of the mixed gels. An increase in aggregation of double helices tends to result in an increase in instability to syneresis, for example on freeze–thaw treatment. Thus in agarose gels the double helices are more aggregated than in κ-carrageenan gels, and they exhibit greater instability to syneresis on freeze–thaw treatment. These two factors present two opposing forces. The first tends to hold the gel network up and to strengthen it,

while the second tends to collapse the gel. For high molecular weight 1,4-linked β-D-glycans there is enough cross-linking effect to counteract the tendency towards syneresis. Thus the net result is a co-gelling activity. However, as the molecular weight of the 1,4-linked β-D-glycan is decreased, the cross-linking mechanism becomes more difficult and aggregation begins to dominate. Thus, as molecular weight decreases, initially a contracted syneresed gel results, and finally a retrograded precipitate forms. This would suggest that addition of low molecular weight 1,4-linked β-D-glycans to agarose, at levels which weaken but still permit gel formation, would result in turbid gels with a greater sensitivity to freeze–thaw syneresis. This is in fact the case. Thus freeze–thaw treatment of 2% agarose results in the release of 10% syneresed liquid, while the addition of 0·5% alkali depolymerised *Cyamopsis tetragonolobus* galactomannan increases the level of syneresis to 50%.

DISCUSSION

It had previously been shown (Dea *et al.*, 1972) that the form of the temperature dependent optical rotation behaviour obtained for the gelation of agarose alone was also observed for non-gelling concentrations. It has now been demonstrated that the complex temperature dependent optical rotation behaviour for gelling mixtures of agarose and galactomannans also occurs for non-gelling systems. This indicates that both these optical rotation transitions originate from molecular effects rather than strain artefacts from gelation. For the interactions between the non-substituted agarose and galactomannans, the general trend between increasing degree of interaction and decreasing galactose content of galactomannan is clear. This is evident from the optical rotation data, in particular the plateau values of net positive contribution to optical rotation change from the galactomannan when mixed with a constant amount of agarose (Fig. 4, Table 2). It is also highlighted by the amounts of individual galactomannans required to gel non-gelling concentrations of agarose (Table 2), and the ability that individual galactomannans have to increase the strength of agarose gels (Fig. 3). It is, however, clear from the data presented that the relationship between degree of interaction and galactose content is not straightforward. Thus there are small but significant differences in the interaction properties of the three galactomannans with galactose content 24%. These galactomannans all exhibit poorer interaction properties than *Caesalpinia spinosa* galactomannan (25% galactose), and the interaction properties of *Ceratonia siliqua* galactomannan are greater than would be expected from its slightly lower galac-

tose content (23%). These perturbations to the relationship cannot be explained by differences in molecular weight between the various galactomannans and are consistent with the differences in fine structure of these galactomannans, and the effect this has on interaction properties, which have been recently reported (Dea *et al.*, 1986*a*, *b*).

Further evidence for the importance of galactomannan fine structure is obtained from the hysteresis behaviour of the temperature dependence of optical rotation for agarose/galactomannan mixtures. Mixtures of agarose with *Ceratonia siliqua* galactomannan give large positive rises in optical rotation on cooling. These optical rotation changes show wide hysteresis loops on reheating, which widen with increase in the ratio of galactomannan to agarose. When the other five galactomannans with galactose contents between 24% and 27% are examined in the same way, the widths of the optical rotation hysteresis loops differ significantly. In particular, the galactomannans from *Caesalpinia pulcherima, Gleditsia triacanthos* (sample 1) and *Gleditsia amorphoides*, which contain 24% galactose, show differing hystereses for the positive rise in optical rotation, which are smaller than the hysteresis behaviour shown by *Caesalpinia spinosa* galactomannan (25% galactose). In addition, the dependence of the width of the hystereses loops on the galactomannan/agarose ratio is less for these galactomannans, and for the *Gleditsia* galactomannans the effect is only slight. The implication of these findings is that *Ceratonia siliqua* galactomannan has a wide molecular spectrum varying from weakly binding to quite strongly binding components, while the *Gleditsia* galactomannans have a narrow spectrum of weakly binding molecules. This is further supported by the fact that *Ceratonia siliqua* galactomannan can be fractionated on a solubility basis to give components differing in galactose content and the ability to interact with agarose, while, at the other extreme, the cold water extract of *Gleditsia triacanthos* seeds interacts with agarose to the same extent as the hot water extract of these seeds. The details of how galactomannan fine structure controls the ability to interact with other polysaccharides is outlined elsewhere (Dea *et al.*, 1986*a*, *b*).

The effect of substitution along the agarose molecule on the interaction with galactomannans was studied by investigating a range of natural agar variants. The types of substitution studied were *O*-methyl ethers (position 6 of D-galactose and position 2 of 3,6-anhydro-L-galactose), *O*-sulphate esters (position 6 of D-galactose and position 2 of 3,6-anhydro-L-galactose), and pyruvic acid ketal on positions 4 and 6 of D-galactose. All of these types and positions of substitution are compatible with the formation of the double helical order conformation of agarose (Arnott *et al.*, 1974*a*), and all the agar variants are gel formers.

From a previous study (Dea *et al.*, 1972) of the interaction of agarose and the carrageenans with galactomannans it could be inferred that substituents along the double helix forming polysaccharide reduce the extent of interaction with galactomannans. This is not because double helix formation is prevented, but rather because the substituents protrude from the outer surface of the double helix, and so reduce the affinity for binding with the extended ribbon ordered conformation of the galactomannan. This effect has been confirmed by the study of these agar variants. For the series of natural *O*-methylated agars examined, there is a clear relationship between the extent of interaction of the agar with *Ceratonia siliqua* galactomannan and the degree of *O*-methylation. This is best shown by following the decrease in the positive contribution to optical rotation change from the galactomannan on cooling agar/galactomannan mixtures, with increase in the degree of *O*-methylation. At *O*-methyl contents equivalent to or greater than one *O*-methyl substituent for every two disaccharide repeating units, no positive contribution to optical rotation on cooling agar/galactomannan mixtures is observed.

Only one agar variant bearing pyruvic acid ketal was examined, and this had a degree of substitution equivalent to one ketal function for every seven disaccharide repeating units. As in the case of the *O*-methylated variants, this substitution led to a decrease in co-gelling activity and in the positive contribution to optical rotation on cooling agar/galactomannan mixtures. As might be expected from the greater size of the pyruvic acid ketal function, in this case the reduction was much greater than that obtained for an equivalent level of *O*-methylation. Similarly, only one sulphated agar variant was studied. This was particularly heavily substituted having, on average, slightly more than one sulphate ester for every disaccharide repeating unit, and in this respect is similar to κ-carrageenan. As expected from the results of the *O*-methylated variants, no positive contribution to optical rotation was obtained on cooling mixtures of sulphated agar and galactomannan.

It is important to note that the absence of a positive contribution to optical rotation is not indicative of an absence of interaction. In fact all the agar variants studied were shown rheologically to interact with galactomannans, although the extent of this interaction decreases with increasing level of substitution. Thus even for heavily substituted agars non-gelling concentrations are gelled by the addition of galactomannans, and addition of galactomannan increases gel strength for gelling concentrations. Similarly, our previous studies (Dea *et al.*, 1972) of the κ-carrageenan/galactomannan interaction indicate that the mechanism involved is the same as that for agar/galactomannan mixtures, although no positive contribution to optical rotation on cooling κ-carrageenan/

galactomannans mixtures is observed. The κ-carrageenan variant furcellaran has also been studied in this way (I. C. M. Dea, unpublished data). This also shows a strong interaction with galactomannans, but no positive contribution to optical rotation on cooling interaction mixtures is observed. It is significant that furcellaran has a sulphate content equivalent to one sulphate ester for every two disaccharide repeating units. For the agar series this is a level of substitution which would not show a positive contribution to optical rotation on cooling a mixture with galactomannan. It is therefore possible that less heavily substituted carrageenans would not only interact better with galactomannans than both κ-carrageenan and furcellaran, but would also show a similar type of optical rotation behaviour as the lightly substituted agars.

In the investigation of the effect of the level of *O*-methylation in the agar on the extent of interaction with *Ceratonia siliqua* galactomannan, two agars fell significantly off the relationship. These two agars also differ significantly from most agars in the relationship between level of *O*-methylation and gel setting temperature (Guiseley, 1970). We conclude from our study that this indicates that fine structural distribution of *O*-methyl groups along the agar molecule, as well as simply the *O*-methyl content, is important in defining the extent of interaction with galactomannans. This is similar to the effect of fine structural distribution of galactose substituents along the galactomannan molecule on the interaction with agarose and xanthan.

In addition to examining the effect of galactose substitution along the β-D-mannan chain of galactomannans on the interaction with agarose, the effect of modification in the 1,4-linked β-D-glycan main chain has been investigated. Three additional 1,4-linked β-D-glycans have been studied: β-D-glucomannan, β-D-glucan and β-D-xylan. All were found to interact with agarose. In the condensed phase, 1,4-linked β-D-mannan (Frei & Preston, 1968), mixed β-D-glucomannan (Chanzy *et al.*, 1982), and β-D-glucan (Dulmage, 1957) adopt very similar extended ribbon ordered conformations, and it is reasonable to assume that, in highly hydrated mixed polysaccharide interacting systems, these similarities in ordered conformation would persist. Although from steric considerations 1,4-linked β-D-xylans can also adopt a similar two-fold ordered conformation, X-ray evidence indicates that they preferentially adopt a three-fold conformation in the condensed state (Settineri & Marchessault, 1965). Soluble 1,4-linked β-D-xylans can, however, adsorb onto cellulose fibres, indicating that they can adopt the ordered two-fold conformation in certain conditions (Gromov *et al.*, 1972). It is therefore a reasonable conclusion that the potential for these different D-glycan main chains to adopt similar overall ordered conformations is an im-

portant determinant in the interaction with agarose, and that changing the configuration at C2 of the mannose residues or replacing the C6 hydroxymethyl group by a hydrogen atom do not forbid the effect. However, the 1,4-linkages for both galactomannans and these alternative β-D-glycans are totally essential for the interaction with agarose. This is demonstrated by the fact that the galactomannan from *Crotalaria mucronata*, which contains a high proportion of non-1,4-linkages in the main chain, and a barley β-D-glucan, which has no long contiguous 1,4-linkages in the main chain, do not interact with agarose.

Insufficient polysaccharides based on these alternative 1,4-linked β-D-glycan main chains were examined, to determine whether degree of substitution has the same effect on the interaction with agarose as it does in the galactomannan series. There were indications from the study that the extent of interaction with agarose was little affected by the degree of substitution by sugar side chains (in the case of the amyloids) or carboxymethyl groups (in the case of carboxymethyl-cellulose). However, differences in molecular weight may have complicated the situation, and more extensive studies are necessary before any firm conclusions on this point can be made. From the results with the substituted β-D-xylans it is clear that substitution by sugar side chains on every main chain sugar residue does not prevent the interaction with agarose. Indeed, the polysaccharide from *Watsonia pyrimidata* corm sacs interacts weakly with agarose despite having every xylose residue substituted, and some even disubstituted. In the case of galactomannans, the available evidence (Dea *et al.*, 1986*a*, *b*) indicates that interaction with agarose and xanthan requires either non-substituted regions of mannan backbone or unsubstituted sides of mannan backbone (i.e. requirement for regions in which every second mannose residue is substituted with galactose). The highest substituted galactomannan studied was that from *Trigonella foenum-graecum*, which has 7% of its mannose residues unsubstituted. It is therefore possible to relate its ability to interact with agarose to the possibility of a limited number of regions of unsubstituted mannan sides in its structure. Totally substituted galactomannans would therefore not be expected to interact with agarose. The ability of totally substituted xylans to interact with agarose is presumably related to the fact that its sugar side chains are attached to C2 and C3 rather than C6.

The final modification of the 1,4-linked β-D-glycan system considered in this study is that of significantly lowering the molecular weight. Some studies have previously been carried out on the effect of mixing low and high molecular weight polysaccharides in gelling systems. Thus the effect of adding segments of κ-carrageenan and ι-carrageenan to gelling concentrations of their respective native parent polysaccharides has been

investigated (Morris *et al.*, 1980). For κ-carrageenan, gel strength is increased by the addition of comparable concentration of segments. The same is true for ι-carrageenan in the presence of sufficient potassium ions (0·5 M KCl) to promote extensive double helix aggregation. At low levels of potassium ions, however, where aggregation is limited, addition of segments significantly weakens ι-carrageenan gels. Mixing segmented and parent carrageenans results in mixed double helical regions, in which segments are bound to a helix forming region of a parent polysaccharide molecule. When there is little or no aggregation of double helices this results in the gel network being inhibited, while when significant aggregation of double helices occurs the gel network is retained. The double helices in agarose gels are highly aggregated, and therefore gel strength is increased by the addition of comparable concentrations of agarose segments (I. C. M. Dea, unpublished data).

The effect of mixing low and high molecular weight polysaccharides has also been previously investigated for mixed gelling systems. Thus κ-carrageenan segments, which form the double helical ordered conformation in solution but which never form gels, interact with the native galactomannan from *Ceratonia siliqua* to form rigid gels (Dea *et al.*, 1972). Similarly, mixtures of agarose segments and *Ceratonia siliqua* galactomannan form rigid gels. In this case the addition of the galactomannan has a stabilising effect, since when alone agarose segments form double helical ordered conformations, which almost immediately aggregate and precipitate out of solution.

In the present study the effect of mixing low molecular weight 1,4-linked β-D-glycans with native agarose and κ-carrageenan has been investigated. The results indicate that the mixtures are antagonistic, and form either weaker gels or, in the extreme, collapsed precipitates. It is concluded that the interaction of ordered extended ribbons of 1,4-linked β-D-glycans with the aggregated double helices of agarose and κ-carrageenan has two opposing effects. The molecular associations lead to more cross-linking of the double helix aggregates, and therefore tend to reinforce gel formation. However, the interaction also results in an increase in the degree of aggregation of the double helices, and this has a tendency to destabilise and collapse the gel. Thus, the first effect tends to hold up and strengthen the mixed gel while the second effect tends to collapse the gel network. For native high molecular weight β-D-glycans there is enough cross-linking effect to counteract the tendency towards gel collapse, and thus the net result is co-gelling activity. However, for low molecular weight β-D-glycans the cross-linking effect is lost, and aggregation with resultant gel collapse dominates. The reason why low molecular weight β-D-glycans have this antagonistic effect, while agarose

and κ-carrageenan segments have a reinforcing effect on the parent polysaccharide gels, is because addition of segments to their parent polysaccharides does not cause any greater increase in the extent of aggregation of double helices than would arise from a simple increase in parent polysaccharide concentration. In contrast, the addition of low molecular weight β-D-glycans results in a major increase in double helix aggregation, and because of the absence of additional cross-linking ability this results in gel collapse.

This study indicates that the co-gelling interaction between agarose and galactomannans can survive major structural variations in the two polysaccharide types. Thus, even at high levels of substitution along the agarose molecule, the ability to interact with galactomannans persists. In addition, the interaction is not unique to the 1,4-linked β-D-mannan main chain. Polysaccharides based on 1,4-linked β-D-glucomannan, β-D-glucan and β-D-xylan main chains also have the ability to interact with agarose. For the double helix forming agarose and the extended ribbon forming β-D-glycans, increasing the substitution along the chains weakens the interaction. In both cases, however, there is evidence that, in addition, the fine structural distribution of substituents along the chain is an important determinant for the extent of co-gelling interaction.

The β-D-glycans studied here are similar in structure to such important skeletal polysaccharides as hemicelluloses and cellulose, suggesting that the association of agarose and the agar variants with the β-D-glycans occur in imitation of natural associations with flexible chains bound in the microfibrillar structure with which they coexist. In addition to cellulose, examples of structural polysaccharides whose interaction with agars could be important *in vivo* are the unsubstituted 1,4-linked β-D-xylans (Turvey & Williams, 1970) and β-D-mannans (Frei & Preston, 1964), which frequently occur in red seaweeds. The structural variations in the agar series not ony affect the adoption of their tertiary double helical structures and therefore the bulk gelling properties, but also materially alter the extent of association with skeletal polysaccharides. In addition, the extent of association is also dependent on variation in structure of, and degree of substitution along, the β-D-glycan main chain. The occurrence of occasional β-1,3-linkages in certain linear, 1,4-linked β-D-xylans from red seaweeds (Cerezo *et al.*, 1971) might therefore be a natural method of modifying the extent of association with agars.

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