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Rheological and Microstructural Evidence for Transient States During Gelation of Kappa-Carrageenan in the Presence of Potassium

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ABSTRACT

Kappa-carrageenan gelation was studied by a combination of electron microscopy and dynamic viscoelastic measurements. There was an initial maximum in the storage modulus during cooling and gel formation of 1% kappa-carrageenan in the presence of potassium. The structure of the transient state was found to consist of a fine network structure, where the junction zones were believed to be double helices. The structure had the characteristics of a true gel but was unstable. When the temperature was lowered aggregation took place and the fine network structure was partly broken down. Ordered superstrands formed which aligned themselves in parallel or were densely packed together.

The degree of aggregation depended on the potassium ion concentration. In the weakest gel formed in 0·01 M KCl, the superstrands did not form a network but were rather dispersed in the fine network structure. In 0·1 and 0·2 M KCl the superstrands formed a three-dimensional network, where aligned superstrands formed the junction zones and branching occurred when superstrands deviated from each other and aligned with new superstrands. In 0·1 M KCl the supermolecular network dominated but in 0·2 M KCl a mixed gel was formed of the fine network and the coarse supermolecular network. The mixed gel gave rise to the firmest gel.

INTRODUCTION

Kappa-carrageenan is a fraction from naturally occurring sulphated galactans from certain species of red algae and has interesting thickening

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and gel forming properties from both an industrial and a physico-chemical point of view.

Gelation of kappa-carrageenan occurs on cooling and has been attributed to a two stage reaction involving the following steps:

$$\text{coils} \rightleftarrows \text{helix formation} \rightleftarrows \text{aggregation}$$

The exact mechanism of this reaction is not yet known. Morris *et al.* (1980) proposed a conformational transition from random coils to intertwined helices, which take the form of soluble clusters or domains and require further association through cation-mediated helix–helix aggregation to develop a cohesive network. Rochas & Rinaudo (1984) found no evidence for intertwined double helices in solution and referred to helical dimers rather than intertwined double helices. They proposed that potassium has a high ionic selectivity for kappa-carrageenan already in the coil state, which controls the net charge of the polymer and promotes the coil–helix transition as well as favouring aggregation by lowering the solubility of helical dimers without the formation of ordered domains of double helices. According to their model, potassium plays no specific role in the crosslinking mechanism except that it affects the solubility of the polymer.

In an earlier paper Smidsrød *et al.* (1980) proposed a model with single helices, and recently Rochas & Landry (1987) proposed the coexistence of single helices with helical dimers at low ionic strength.

The coil–helix transition has been the subject of most studies related to carrageenan gelation. It is well established that the transition temperature is highly dependent on the concentration and type of cation. Of the cations commonly associated with kappa-carrageenan potassium is far more efficient than either calcium or sodium (Rochas & Rinaudo, 1980).

Far less is known about the helix–aggregation reaction. Information has mainly been obtained from hysteresis in optical rotation between cooling and heating curves and from calorimetric and conductivity measurements during melting of the aggregated structure (Morris *et al.*, 1980; Rochas & Rinaudo, 1982, 1984).

The earlier models proposed for the network formation are not in agreement and very schematic pictures of the gel structure have been given. Recent results from Day *et al.* (1988) indicate a more complex mode of aggregation due to phase separation, with aggregated molecules of preferentially higher molecular weight distributed throughout a solution of dissolved molecules.

The work reported here provides new information about the complexity of kappa-carrageenan gelation and details about the type of supermolecular structures formed at various concentrations of potassium ions. For the purpose of this study a combination of electron microscopy and viscoelastic measurements at different states of gelation was made.

Previously reported rheological studies have only dealt with the melting behaviour of kappa-carrageenan gels and not with events taking place during cooling and gel formation (Morris & Chilvers, 1983; Rochas & Rinaudo, 1984). During melting a continuous decrease in the storage or shear modulus with increasing temperature was observed.

Electron microscopy provides a valuable tool for gaining knowledge about supermolecular structures formed during the aggregation and gelation of biopolymers (Hermansson, 1988). For the study of kappa-carrageenan conventionally used preparation techniques have to be critically evaluated and further improved. The two most commonly used techniques for biological macromolecules are the 'negative staining' and the 'mica' techniques. Negative staining has not been considered for this study since the technique is based on the addition of a contrasting solution of heavy metal ions, which may influence the highly ion-sensitive reactions involved in the gelation of kappa-carrageenan.

The 'mica technique' generally means that a biopolymer solution is mixed with glycerol and sprayed onto a freshly cleaved mica surface (Tyler & Branton, 1980). The most common way of using this technique to study carrageenan gelation involves several hazards. First, it has recently been shown that glycerol has a pronounced effect on the gelation of kappa-carrageenan in the presence of potassium (Nishinari & Watanase, 1987; Gekko *et al.*, 1987). Second, shear-induced damage of supermolecular structures can easily occur during spraying. Third, sequential adsorption of components takes place when the drop of solution slides and dries on the mica surface, which makes it impossible to determine the distribution of components in a multiphase system. Further, it is difficult to control the temperature adequately, especially at elevated temperatures.

To overcome these difficulties the mica sandwich technique developed by Mould *et al.* (1985) was adopted. A thin layer of solution is gently placed between two freshly cleaved mica surfaces, a method that reduces the shear-induced damage. Drying can be obtained by rapid freezing followed by freeze drying, making it unnecessary to add glycerol or other additives during preparation of the sample for electron microscopy. The sandwich technique also offers the possibility of conditioning the sample at any temperature and then 'freezing' the structure at that temperature. This has made it possible to study different structural states during gelation of kappa-carrageenan.

MATERIALS AND METHODS

Samples of kappa-carrageenan from *Eucheuma cottonii* type III were purchased from Sigma Chemicals (St Louis, MO, USA). The pure potassium and sodium forms were prepared by ion exchange with a commercial ion exchange resin (AG 50W-X8, Bio-Rad) according to the procedure described by Morris & Chilvers (1983). The solutions were concentrated in a rotary evaporator at 70°C and freeze dried. If not otherwise stated, the pure potassium form was used in this study.

Rheological measurements were made in a Bohlin VOR rheometer system with the C14 measuring system, by sequential oscillatory measurements every 30 s at a frequency of 1 Hz and a strain of 0·003 if not otherwise stated. A 1% solution of kappa-carrageenan was heated at 98°C for 15 min, poured into the rheometer and cooled to 90°C. A jobstream program was used for the measurements including the following sequences: (1) a temperature decrease of 1·5°C/min from 90 to 20°C; (2) measurements for 1 h at 20°C; (3) a change in the frequency from 0·01 to 10 Hz at 20°C; (4) a strain sweep from 0·0008 to 0·08 at 20°C or instead sequence (1) and (2) followed by a temperature increase from 20 to 90°C to study the melting process. Four replicates were made. The effect of factors such as geometries, heating rates and intervals of sampling were investigated in preliminary experiments.

Samples were prepared for electron microscopy by cleaving a piece of mica (14 × 14 mm) and placing 2 μl of a solution of carrageenan (0·1 mg/ml), cooled to the required temperature, on the newly formed surface. The other piece was gently replaced on top causing the drop of solution to spread between the two plates in a thin layer approximately 10 μm thick. The two mica plates were somewhat displaced in order to facilitate their separation. This procedure was performed in a thermostated humid cupboard, whereafter the sample was allowed to equilibrate for 5 min. It was then plunged into liquid propane or liquid nitrogen and the pieces of mica were separated at a low temperature. The sample was freeze dried on a Balzers BA 360 freeze etching unit. The initial temperature of the table was −100°C, whereafter the table temperature was kept constant at −50°C for 1 h. The sample was freeze dried for 2 h to a pressure of 2×10^{-6} torr and rotary shadowed with Pt/C at 4°. Replicas were examined in a JEOL 100CX-II at an accelerating voltage of 80 kV. Several preparations were made from every carrageenan sample and micrographs were chosen out of hundreds.

Comparative studies were also made with samples cooled to room temperature by glycerol drying using both spraying and the mica sandwich technique according to the procedures described by Mould *et* *al.* (1985). As has been stated above, glycerol cannot be used for studies of the aggregation process. However, once superstrands were formed, glycerol did not seem to have any significant effects on the potassium-induced structures. The results from the comparative studies clearly showed that spraying caused damage to supermolecular assemblies and fine network structures.

RESULTS AND DISCUSSION

Transient states during gelation

The initial phase of gelation was followed by oscillatory measurements as the temperature was reduced from 90 to 20°C. Figure 1 shows the storage modulus G′, the loss modulus G″ and the phase angle delta of 1% kappa-carrageenan in 0·1 M KCl. On cooling, a pronounced maximum in G′ was observed. Such an initial transition in the rheological behaviour of kappa-carrageenan has not been reported previously. The maximum was reproducible and, as can be expected for phase transitions, the changes

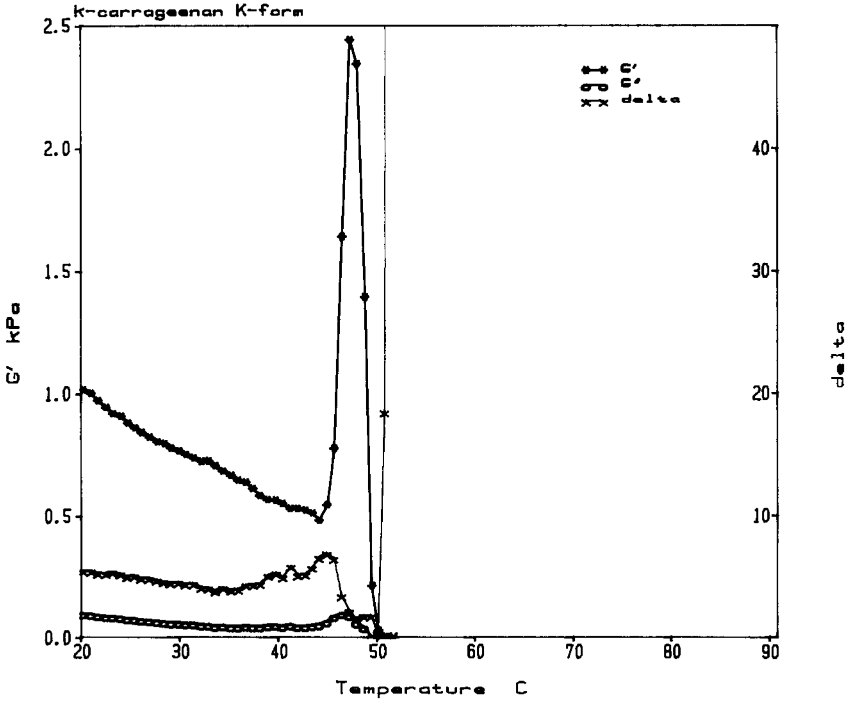

Fig. 1. The storage modulus G′, loss modulus G″ and phase angle delta as a function of the temperature during cooling of 1% kappa-carrageenan in 0·1 M KCl.

in G′ were followed by maxima in the loss modulus and in the phase angle. On gelation, there is a very sharp decrease in the phase angle to less than 5° and in Fig. 1, as low as 0·4°. This decrease is very distinct and its temperature highly reproducible. It was therefore taken as a measure of the gelation temperature. The recorded temperature (50·4°C) coincides well with the temperature of the coil–helix transition as reported by Rochas & Rinaudo (1980). The rheological data thus support the general view that the coil–helix transition is the first step in the gelation process. Plashchina *et al.* (1986) reported different results, since in their study gelation took place above the transition temperature. They reported gelation temperatures at 80°C for the potassium form in 0·2 M KCl and 66°C in 0·1 M KCl, which is far above the results obtained on cooling in this study. A possible reason for their results could be that the carrageenan was not fully dissolved to the coil state before the start of their experiments (see Fig. 9).

The transient state of the potassium form of kappa-carrageenan is a solid gel structure with a high storage modulus and a very low phase angle. This cannot be caused by soluble clusters of double helices or by helical dimers in solution. To gain an understanding of the structural changes taking place during the initial phase of gelation, samples were prepared for electron microscopy at temperatures slightly above and below that of the peak maximum, as well as after cooling to 20°C. As a reference we wanted a solution with random coils. This was considered difficult to obtain with the potassium form of kappa-carrageenan. For this purpose the pure sodium form was dissolved in distilled water and then heat treated. Figure 2 shows a micrograph of random coils at a magnification of 150 000x. The coils are very fine and at the limit of the resolution. It is probably not possible to learn anything at all from the print. Thus we cannot analyse or detect the distribution of single random coils of kappa-carrageenan by electron microscopy.

The structure of a transient state in 0·1 M KCl is shown in Fig. 3. A fine and regular network structure is clearly seen. The strands have a periodicity and are interpreted as helical dimers, which could very well be double helices. Instead of forming clusters the ordered domains fill out the whole volume giving rise to a solid and elastic network structure. This structure is not stable and when the temperature is further decreased an aggregation process is initiated. Figure 4 shows the structure at a temperature below that of the peak maximum. The fine network structure is partly broken down and rigid rods have formed, which are believed to consist of two helical dimers tightly packed together.

The dimers can often be seen aligned in parallel, but also twisted

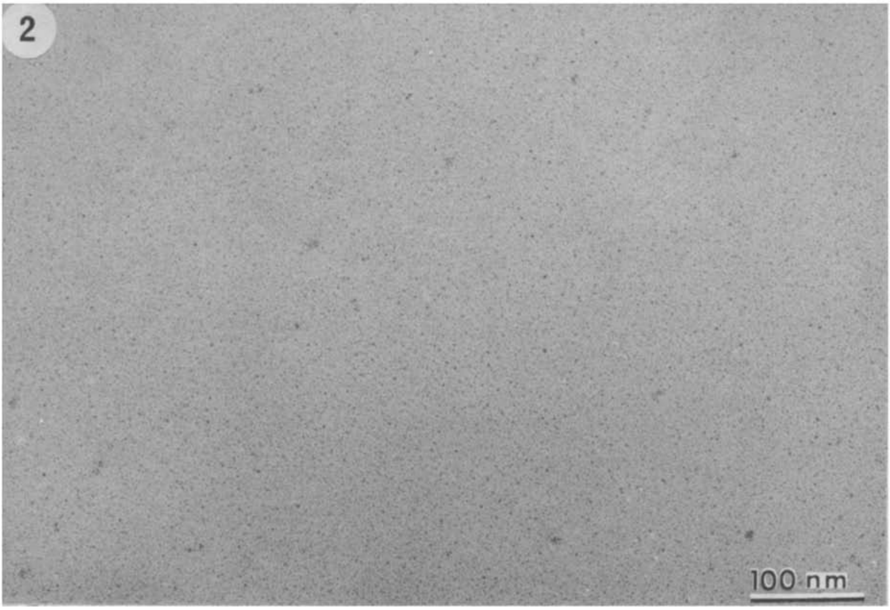

Fig. 2. Random coils on mica.

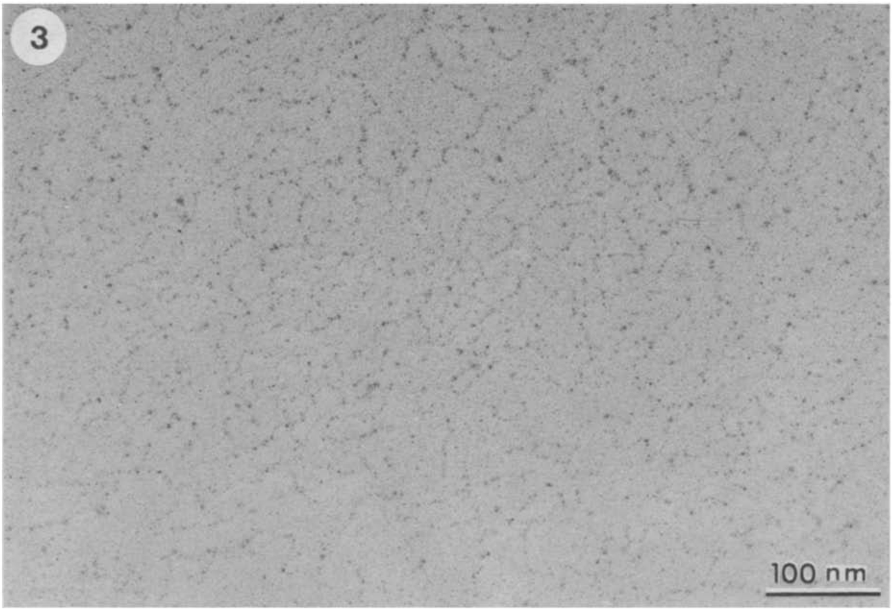

Fig. 3. Transient state of kappa-carrageenan in 0·1 M KCl.

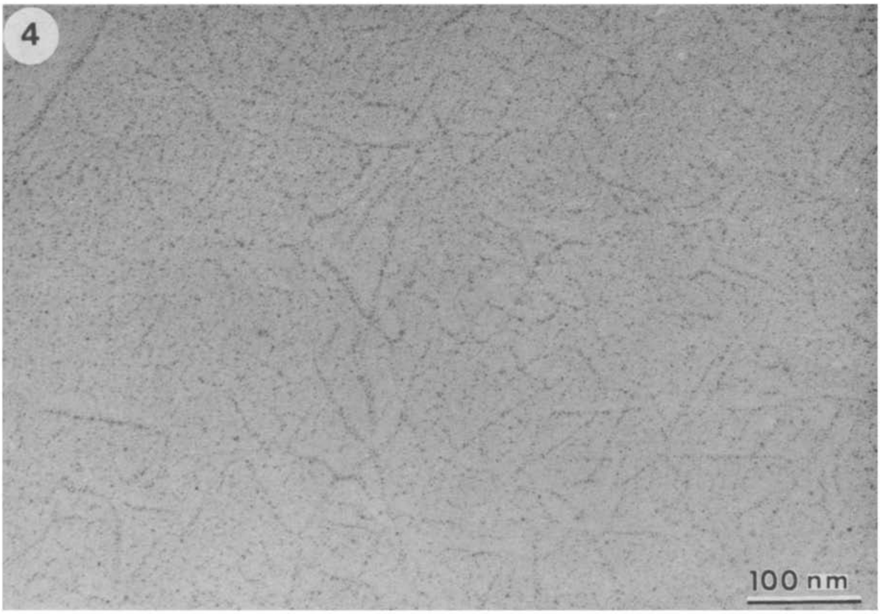

Fig. 4. Initial aggregation of kappa-carrageenan in 0·1 M KCl.

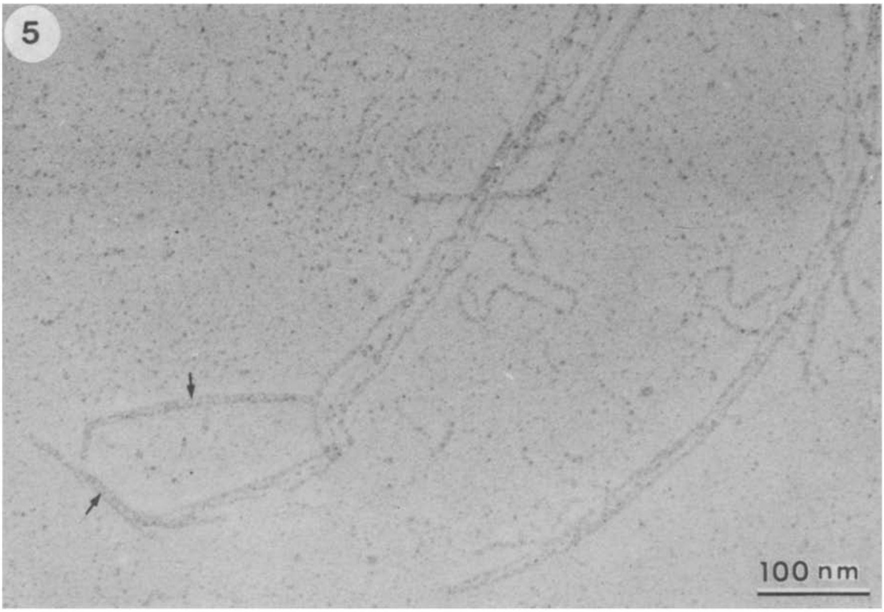

Fig. 5. Completed aggregation of kappa-carrageenan in 0·1 M KCl.

around each other. The destruction of the fine network structure is probably the cause of the drop in the storage modulus. As the temperature is further decreased the superstrands continue to aggregate, building up a coarser network structure, and the storage modulus starts to increase again until this process is completed. Figure 5 shows details of the structure at 20°C. Part of the fine network structure still remains and here it may be appropriate to talk about clusters of ordered domains. On the supermolecular level, kappa-carrageenan has aggregated to form very long strands. The arrows point to regions where superstrands are densely packed together with, as it seems, some degree of periodicity. In other parts, it is clearly seen that superstrands, often three, align in parallel with a small distance in between. It is interesting to observe that the potassium-induced superstrands are not aggregated at random but form quite ordered and rigid assemblies in spite of their complexity.

The nature of junction zones has often been explained in terms of double helices and the network as being induced by linking residues incompatible with helix formation as reviewed by Morris (1986). This cannot be the reason for network formation on the supermolecular level. Some details of supermolecular junctions are shown in Fig. 6(a) and (b). Junction zones are formed by the alignment of superstrands either in the form of densely packed superstrands, as shown in Fig. 6(b), or as a parallel alignment of two or three superstrands, as shown in Fig. 6(a). A network is formed when aligned superstrands deviate from each other and align themselves with new superstrands.

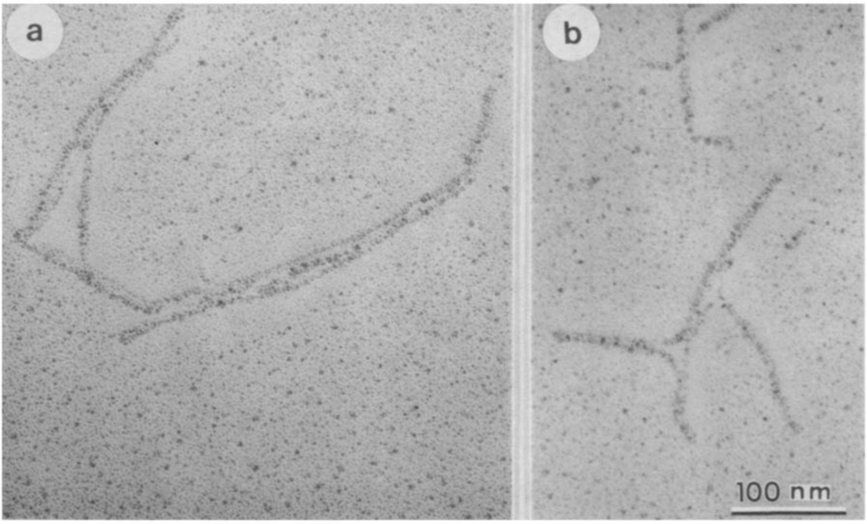

Fig. 6. Details of junction zones of kappa-carrageenan in 0·1 M KCl.

Effect of potassium concentration

Rheological and structural studies of kappa-carrageenan were made in the presence of 0·01, 0·1 and 0·2 M KCl. The lowest potassium ion concentration chosen was slightly above that necessary for the coil–helix transition, regardless of the ionic form of kappa-carrageenan. The total ionic concentration is somewhat higher for the potassium form, since the activity of the counter ions needs to be taken into account. According to the results of Rochas & Rinaudo (1980) the critical potassium concentration for coil–helix transition is approximately 7 mM at 20°C.

Figure 7 shows the changes in the storage modulus G′ of 1% kappa-carrageenan in 0·01, 0·1, and 0·2 M KCl during cooling from 90 to 20°C and a holding time of 1 h at 20°C. The storage modulus of kappa-carrageenan in 0·01 M KCl had no pronounced initial maximum but

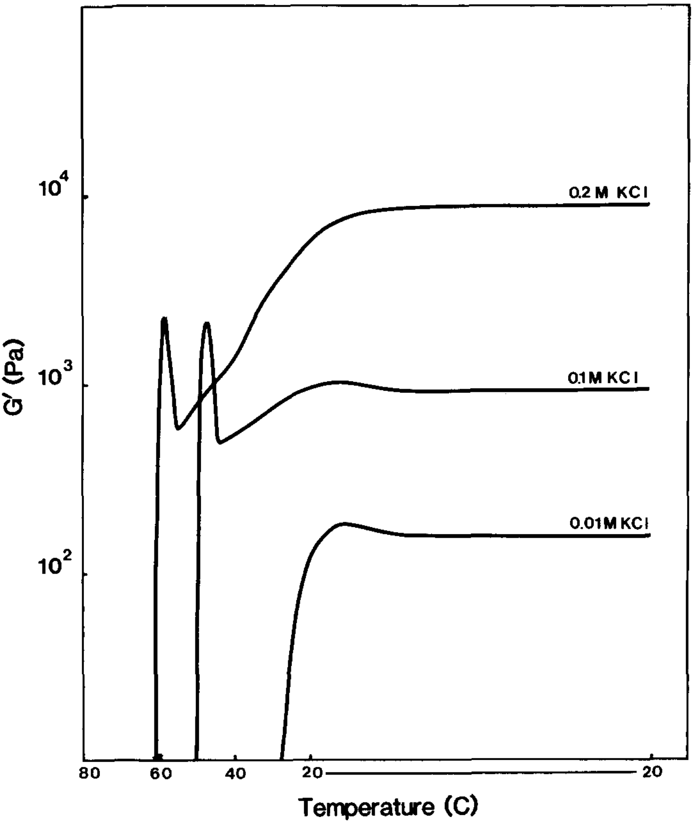

Fig. 7. The storage modulus G′ as a function of temperature for 1% kappa-carrageenan in 0·01, 0·1 and 0·2 M KCl.

rather a small shoulder. The gelation temperature recorded was 28°C. There was a sharp maximum in the storage modulus at 0·1 M KCl as discussed above. At 20°C, the storage modulus stabilised at a value below that of the peak maximum. The gelation temperature was recorded at 50·4°C. There was also an initial maximum in the storage modulus at 0·2 M KCl, but then the storage modulus increased to a value above that of the peak maximum and a firmer gel than that of the transition state was formed. The gelation temperature recorded was 61·5°C.

Although there was a pronounced difference in the gel strength between the firmest gel, formed in 0·2 M KCl, and the weakest gel, formed in 0·01 M KCl, the storage moduli for all three gels were fairly independent of frequency as shown in Fig. 8. Thus even the weakest gel shows a frequency behaviour typical of a true gel and not of an entanglement structure. The linear regions of the gels differed considerably. The gels formed in 0·1 and 0·2 M KCl had a narrow linear region and were only linear to a strain of 0·4% (0·004), whereas the gel formed in 0·01 M KCl was linear to a strain of 4%.

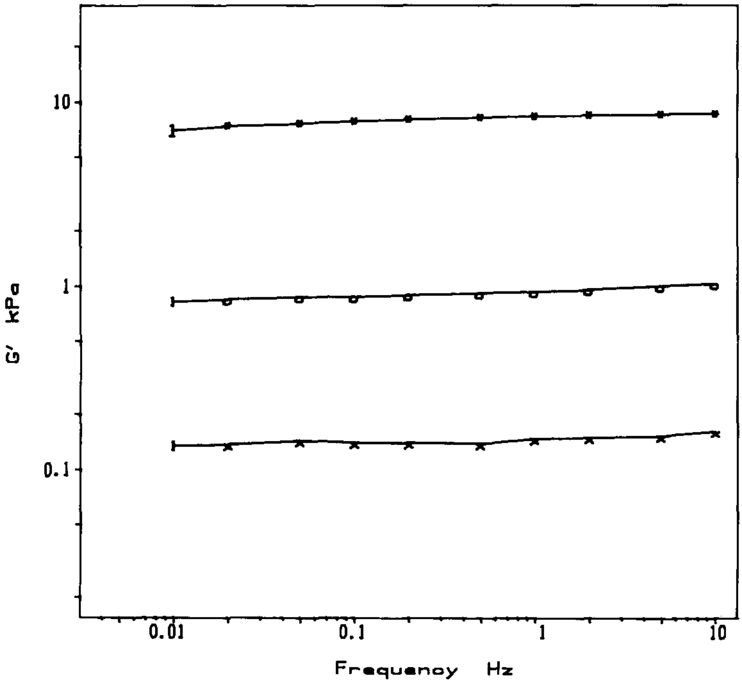

Fig. 8. The storage modulus G′ as a function of the frequency for 1% kappa-carrageenan in, (— × —), 0·01 M KCl; (– □—), 0·1 M KCl; and (—*—), 0·2 M KCl.

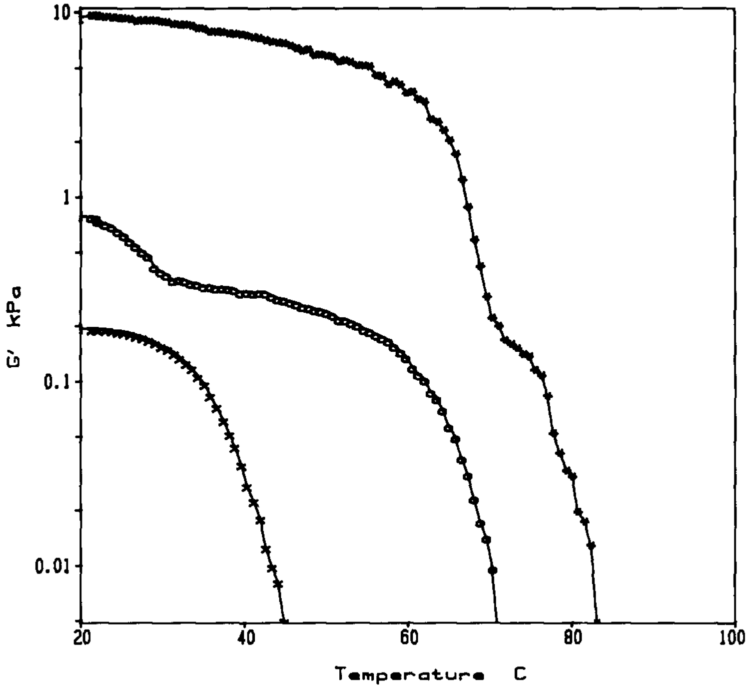

Fig. 9. The storage modulus G′ as a function of temperature during heating of 1% kappa-carrageenan in, (— × —), 0·01 M KCl; (—□—), 0·1 M KCl; and (—*—), 0·2 M KCl.

The melting curve differed considerably from the heating curves and formation of the gel structure. Figure 9 shows the changes in storage modulus when the temperature was increased from 20 to 90°C. As expected, there is a pronounced hysteresis between the cooling and the heating curves. There are no local maxima in the melting curves but their shapes, especially that of the gel formed in 0·2 M KCl, indicate a complex gel structure.

Some interesting observations can be made from the corresponding changes in the phase angle shown in Fig. 10. The phase angle of the gel made in 0·2 M KCl showed a pronounced maximum around 68°C, before the gel was fully melted at 83°C. This was not the case for the gel made in 0·1 M KCl, where the phase angle increased sharply at 70°C. The difference in the melting behaviour indicates a difference in the gel structure between these gels. There was also a maximum in the phase angle for the weakest gel made at 0·01 M KCl, before the structure was fully melted. The phase angle was generally more unstable during heating and melting than during cooling and gel formation.

In order to obtain further information about structural differences

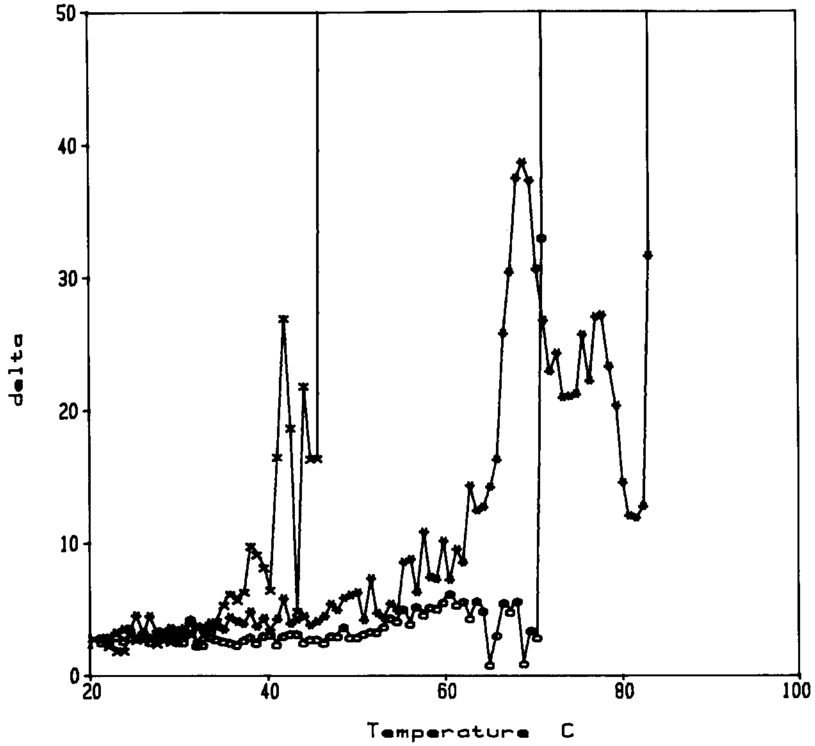

Fig. 10. The phase angle delta as a function of temperature during heating of 1% kappa-carrageenan in, (— × —), 0·01 M KCl; (—□—), 0·1 M KCl; and (—∗—), 0·2 M KCl.

between gels made at various KCl concentrations, a microstructure analysis was made, which can explain some of the differences in their rheological behaviour. Figure 11(a)–(c) shows the structures of kappa-carrageenan in 0·01 M KCl at a magnification of 100 000x. The dominant part of this structure is a fine network structure similar to that of the transient state shown in Fig. 3. Some superstrands can also be seen. However, the superstrands do not form a continuous network structure but are only partly aggregated and dispersed in the fine network structure. The formation of superstrands may be the reason for the shoulder in the storage modulus depicted in Fig. 7. The micrographs thus indicate that the ordered fine network structure of helical dimers predominates and that aggregation into superstrands is suppressed at low potassium concentrations close to that required for the coil–helix transition.

The microstructure changed completely when the KCl concentration was increased to 0·1 M, as seen from Fig 12(a)–(c). The fine network

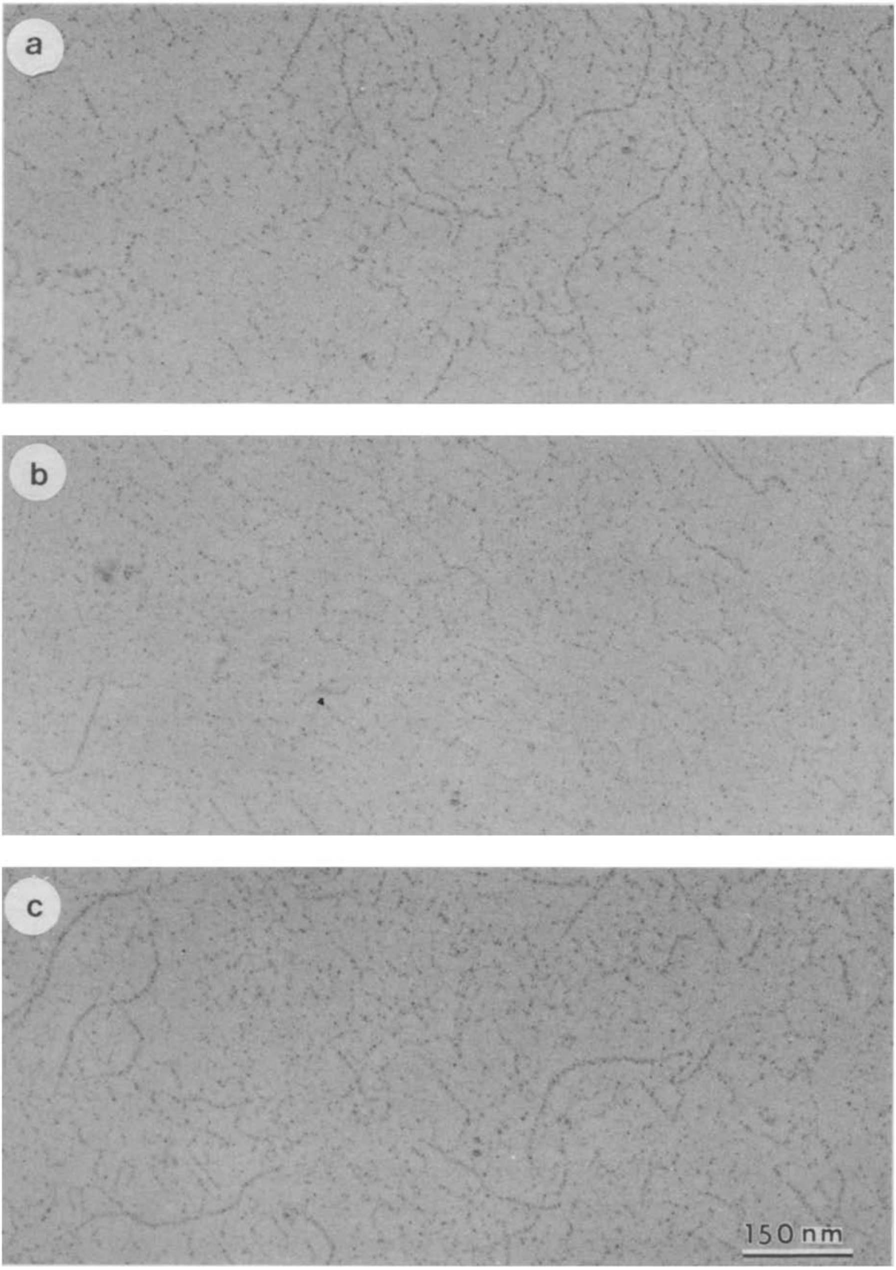

Fig. 11(a)–(c). Structures of kappa-carrageenan in 0·01 M KCl.

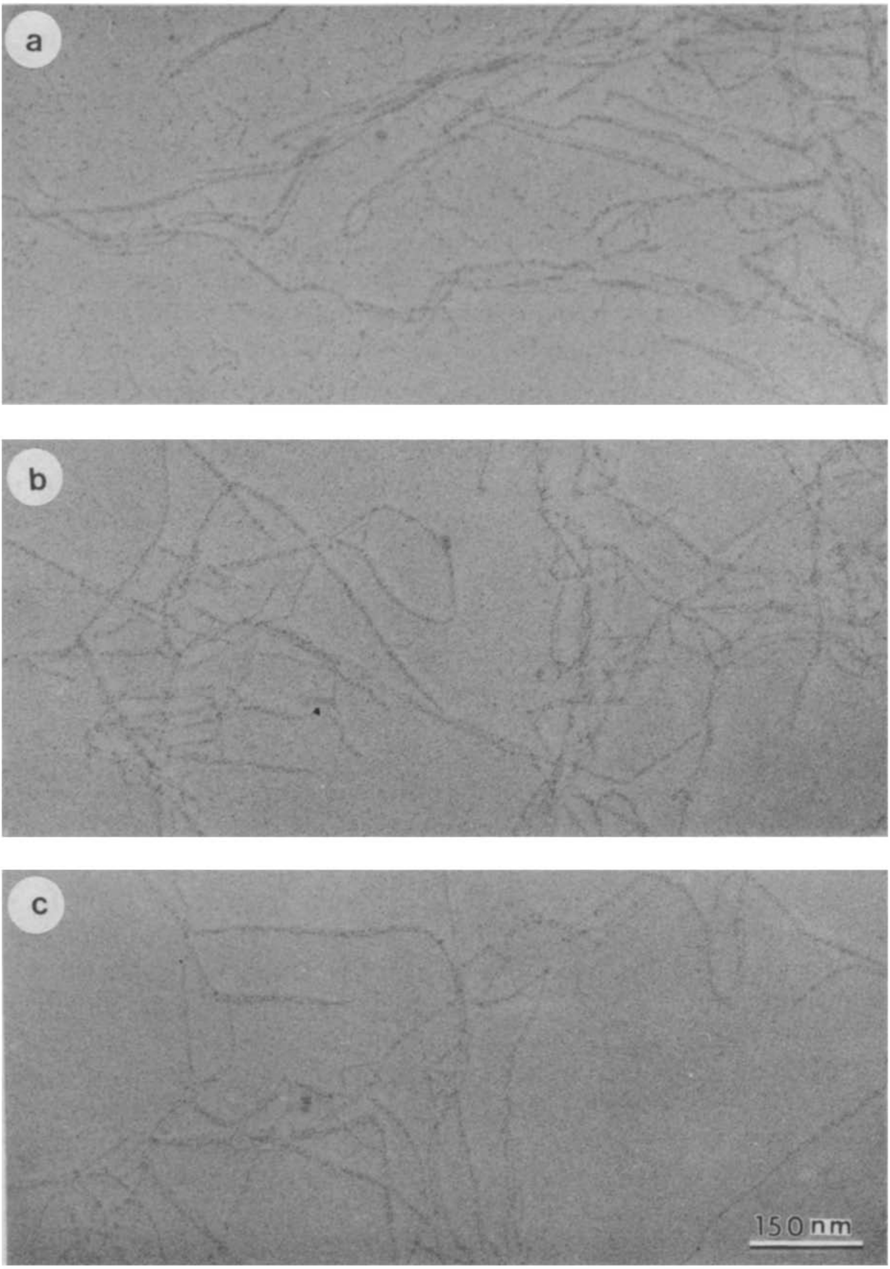

Fig. 12(a)–(c). Structures of kappa-carrageenan in 0·1 M KCl.

structure has almost completely disappeared. Fragments of this structure can be seen in Fig. 12(a) and in Fig. 5 at a higher magnification, but not at all in Fig. 12(b) and (c). Instead, aggregated superstrands make up an irregular and rather stiff network structure.

Figure 13(a)–(c) shows structures formed in 0·2 M KCl. In this case, there is a mixed network structure consisting of a network of aligned superstrands and the fine network structure. Both types can form continuous networks spanning the whole sample, which may be the reason for the firm gel structure. It is possible that the higher salt concentration stabilises the fine network structure protecting it from disruption and also strengthening it, but once superstrands have formed, the higher salt concentration favours further alignment and aggregation into a network structure. The results also indicate that the maximum in phase angle upon melting is due to a phase transition and melting of the fine network structure present in the gels made in 0·2 M and 0·01 M KCl, but not to the same extent in the gel made in 0·1 M KCl. The difference in the linear regions between the gels can be attributed to the supermolecular network structure present in 0·1 and 0·2 M KCl, but not in 0·01 M KCl.

By comparing the micrographs shown in Figs 11, 12 and 13, differences between structures formed in 0·01, 0·1 and 0·2 M KCl can be identified and used to explain differences in rheological behaviour. In order to obtain detailed information on the molecular and supermolecular levels, it was necessary to work with techniques that required more dilute systems than those used in the gelation studies. The proportions between the different types of structures may differ due to concentration, but the characteristic differences due to salt concentration are believed to be the same. From corroborative studies on containing kappa-carrageenan with the ‘thin sectioning technique’, it was found that 2 and 3% kappa-carrageenan gels had structures composed of dense fine network structures and loose networks of superstrands that varied in proportion to the concentration of potassium. This technique is being further developed since at present the contrast is low.

If the results obtained in this study are compared with the schematic models proposed by Morris *et al.* (1980), and Rochas & Rinaudo (1984), it seems as if both models cover a part of the complex gelation phenomenon of potassium-induced gelation of kappa-carrageenan. The formation of ordered domains, as suggested by Morris *et al.* (1980), agrees with the formation of the fine network structure of the transient state at high KCl concentrations and with the formation of the structure formed at very low KCl concentrations. The difference is that our results show that this structure can span the whole volume and form a true gel under special conditions rather than forming soluble clusters. The model

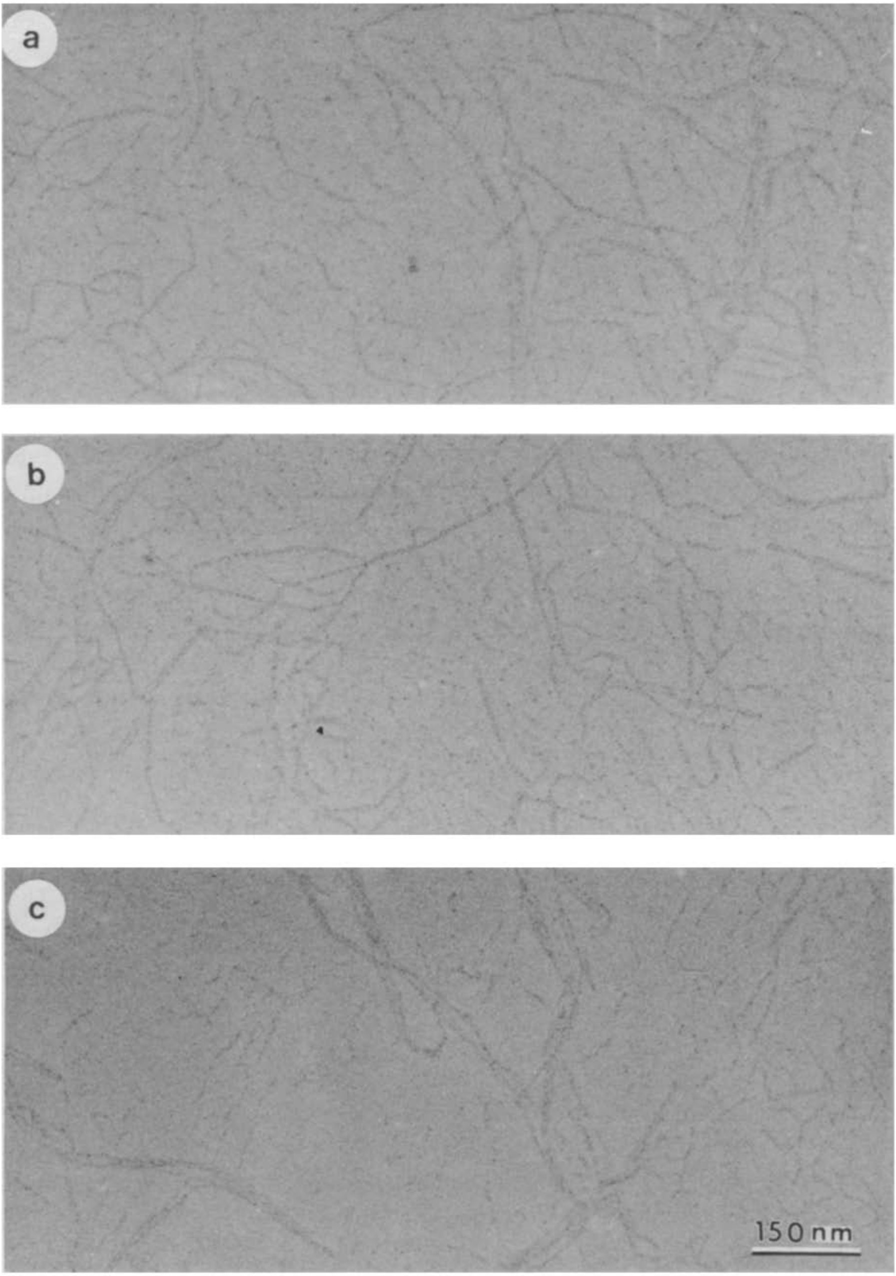

Fig. 13(a)–(c). Structures of kappa-carrageenan in 0·2 M KCl.

of Rochas and Rinaudo stresses the fact that potassium favours the aggregation of helical dimers into superstrands, even though the nature of the superstrands is not being considered in any detail.

It should be pointed out that the type of stiff superstrands shown here are specifically induced by potassium, and that quite different supermolecular assemblies may be induced by other cations. The effect of the type of cations on the structure and rheological properties of kappa-carrageenan will be considered in another paper.

CONCLUSIONS

A transient state of kappa-carrageenan in the presence of potassium has been found both by structural and rheological measurements. The transient structure consists of a fine network, where the junction zones are believed to be double helices. It has the characteristics of a true gel but is unstable and formed only within a narrow temperature range or at very low salt concentrations. When the temperature is lowered, an aggregation process is initiated, which results in a marked drop in the storage modulus. Initially, helical dimers are believed to associate into rigid rods, which then align themselves into long supermolecular assemblies where two or more superstrands are aligned in parallel, with a small distance in between, or tightly packed in some periodic manner.

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