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Tokuya Harada

Yoshimi Kanzawa

Kazuko Kanenaga

Atuo Koreeda

Akira Harada

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ELECTRON MICROSCOPIC STUDIES ON THE ULTRASTRUCTURE OF CURDLAN AND OTHER POLYSACCHARIDES IN GELS USED IN FOODS

Tokuya Harada, Yoshimi Kanzawa, Kazuko Kanenaga, Atuo Koreeda[1] and Akira Harada[2]

Kobe Women's University, Suma-ku, Kobe-shi 654
[1] Institute of Scientific and Industrial Research, Osaka University, Mihogaoka, Ibaraki-shi 567
[2] Faculty of Science, Osaka University, Machikane-yama, Toyonaka-shi 560
Japan

Abstract

Curdlan gels form either by neutralization of alkaline solutions or by heating aqueous suspensions. The former gels consist of long microfibrils, 20 to 25 nm wide, made up of subunits, 2-3 nm wide. The microfibrils disintegrate on sonication into fibril units which on heating loosen and release thinner elementary fibrils, 1-3 nm wide.

Heated gels are composed of electron-dense structures in pseudocrystalline form. Preparations having their average degree of polymerization ($\overline{DP}$n) 200, are unable to form gels.

Agar, κ- and ι-carrageenans, and konjac glucomannan gels formed by cooling of heated sols have structures similar to gels produced by neutralization of their alkaline solutions. Both neutralized and heat-induced gels are composed of long microfibrils, about 5-25 nm wide. Viscous solutions of λ-carrageenan, scleroglucan, succinoglycan, xanthan gum, pullulan, and dextran are composed of shorter microfibrils, 1-2 nm wide. Sodium salts of κ-carrageenan, alginate, gellan gum, and low-methoxyl pectin form gels in the presence of potassium or calcium ions. The microfibrils in these gels are considerably longer and/or wider than in the sols. Locust bean gum does not gel alone but yields gels containing fibrous or globular forms when mixed in solution with xanthan gum or κ-carrageenan and potassium ions. Fibrous structures of amylose and amylopectin in starch gels appear to be similar. Short-chain amylose ($\overline{DP}$n 57 and 78) consists of branched microfibrils about 10 nm wide.

Molecular association resulting in the formation of long and/or wide microfibrils in rod-like or globular forms is supposedly one of the prerequisites for polysaccharides to form gels.

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Direct inquiries to T. Harada
Telephone number: 81 78 731 4416
Telefax Number: 81 78 732 5161

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Introduction

Polysaccharides are important polymers present in foods as sources of energy and dietary fibre and influence structure and flow properties. Starch and pectin are widely distributed in vegetables and fruits. Agar, carrageenan, and alginate - acidic polymers in seaweeds - are used as food additives. A glucomannan in konjac (*Amorphophalus konjac*), cultured in Japan, is used as staple food. Many kinds of β-D-glucans with mainly (1→3)- and some (1→4) and/or (1→6)-linkages are present in various kinds of mushrooms grown in oriental countries, particularly Japan. Microbial polysaccharides are also produced as food additives.

Curdlan, a bacterial polysaccharide, is used at concentrations of about 0.05% to 5% to solidify some food products. It improves the texture of various foods including soybean curd (tofu), sweet bean paste (yokan), boiled fish paste (kamaboko), Japanese noodles (udon), sausages, jellies, and jams [12]. Some new applications to foods have recently been developed by Takeda Chemical Ind. Ltd., particularly to foods subjected to retorting and/or freezing, *e.g.,* thin-layered gels flavoured with honey, strawberries, noodle-shaped soy-milk gel, frozen konjac-like gelled foods, and jellyfish-like gel foods packaged in retort pouches. Some kinds of foods which contain curdlan such as quick serve or snack rice are now commercially available. Moreover, curdlan has a potential to be used in binders, coagulating agents, and films on an industrial scale.

In Japan, agar is used in sherbets and sweet bean paste (yokan), and κ-carrageenan is used in desserts and canned meats. Alginate and low-methoxyl pectin are used in a variety of gel products such as instant puddings, fruit gels, and dessert gels. Viscous polysaccharides are used as emulsifiers, stabilizers, and thickening agents in many foods. For example, xantham gum is used in salad dressings, ice cream, etc. Pullulan is utilized in edible films. Starch is an important thickening and binding agent and is used widely in the production of puddings, soups, sauces, salad dressings, boiled fish paste (kamaboko), and other foods.

Conformational studies of these polysaccharides have been done mainly using x-ray diffraction analysis. Nuclear magnetic resonance (NMR) analysis using ^{13}C and electron microscopy are very helpful in such studies. This paper reports the results of electron microscopical analysis of the ultrastructures of polysaccharide gels, particularly curdlan, used in foods.

Table 1.

The polysaccharides used in this study and their origin

Chemical origin:	Commercial origin:
Curdlan from *Alkaligenes faecalis* var. *myxogenes* 10C3K	Harada
Succinoglycan from *Alkaligenes faecalis* var. *myxogenes* 22	Wako Pure Chem. Ind. Harada
(1➔3)-β-D-glucan (DPn 131 and 49)	Takeda Chem. Ind., Ltd.
Agar from *Gracilaria verrucosa*	Ina Food Co. Ltd.
κ-Carrageenan from *Euchuma cottonii*	Ina Food Co. Ltd.
ι-Carrageenan from *Iridea laminaioides*	Ina Food Co. Ltd.
λ-Carrageenan from *Euchuma spinosum*	Ina Food Co. Ltd.
Konjac glucomannan from *Amorphophalus konjac*	Prepd. from konjac available on market
Xanthan gum from *Xanthomonas campestris* (Kelco Co. Ltd.)	Iwata Chem. Co. Ltd.
Scleroglucan from *Sclerotium glucanicum*	Pillsbury Co. Ltd.
Pullulan from *Pullularia pullulans*	Hayashibara Biochem. Institute
Dextran from *Leuconostoc mesenteroides*	Wako Pure Chem. Ind.
Pectin 3450-NH-95J (Unipectin France)	Meidiya Co. Ltd.
Gellan gum from *Pseudomonas elodea* (Kelco Co. Ltd.)	Ina Food Co. Ltd.
Locust bean gum from *Ceratonia siliqua*	Nitta Gelatin Co. Ltd.
Starch granules	Hayashibara Biochem. Institute
Amylose from corn, cassava, potato	Hizukuri
Short chain amyloses (DPn 18, 57, 78, 117)	Hayashibara Biochem. Institute

Curdlan was discovered in 1966 by Harada *et al.* [7, 11] as a polysaccharide capable of forming gels on heating in water. Earlier studies on curdlan have been reviewed by Harada [7, 8] and Kasai and Harada [21]. Curdlan is produced by a mutant strain of a soil bacterium, *Alcaligenes feacalis* var. *myxogenes* [11]. It may be used to solidify liquids or to bind fine powders not only in foods but also in other industrial applications [12]. It is produced commercially by Takeda Chemical Ind. Ltd. (Nihonbashi 2-12-10, Chuo-ku, Tokyo 103).

Characteristics of the Polysaccharides under Study

The origins and structures of the polysaccharides dealt with in this study are shown in Tables 1 and 2, respectively.

Pure sodium forms of κ-carrageenan, xanthan gum, and pectin were prepared in the following way [20]: Commercial preparations were dialyzed against water to remove contaminating inorganic salts. The cations present in the dialyzed preparations were removed by passing the preparations through a column of Amberlite IR-120 resin in H^+-form. Sodium forms were prepared by neutralizing the solutions in H^+-form using sodium hydroxide, centrifuging the neutralized polymer, dehydrating the precipitate using acetone, and drying it in air.

The polymers under study have the following properties:

Curdlan is insoluble in water but swells in it. It is soluble in alkaline solutions, formic acid, and in dimethyl sulfoxide. Curdlan forms two types of gel [8] which will be dealt with in the Experimental Techniques Section 3. Agar forms gels after its aqueous suspensions are solubilized by heating above 90°C and then cooled below 40°C. In carrageenan, the contents of sulfate and anhydrosugars differ depending upon the kind of preparation. Solubility in water increases with the increase in the sulfate content and the decrease in the anhydrosugar content. λ-Carrageenan is unable to form gels and is viscous in aqueous solutions but κ-carrageenan forms gels in the presence of potassium ions. Konjac glucomannan is able to form gels when its aqueous suspensions are heated in the presence of calcium ions. Agar, κ-carrageenan in the presence of potassium ions, and konjac glucomannan also form gels when their alkaline solutions are dialyzed. Neutralized gels seem to be the same

Table 2.

Structures of the polysaccharides under study

Poly-saccharide:	Monosaccharide building units:	Linkages:
Curdlan	D-Glucose	(1➔3)-β-
Succinoglycan	D-Glucose 7	(1➔4)-β-, main chain (1➔3)-β-, side chain (1➔6)-β-, branching point
	D-Galactose 1	(1➔4)-β-, main chain
	Pyruvic acid	
	Succinic acid	
	Acetic acid	
Agar	D-Galactose	(1➔4)-β-
	3,6-Anhydro-L-galactose sulfate	(1➔3)-α-
κ-Carrageenan	D-Galactose-4-sulfate	(1➔4)-β-
	3,6-Anhydro-D-galactose	
ι-Carrageenan	D-Galactose-4-sulfate	(1➔4)-β-
	3,6-Anhydro-D-galactose-2-sulfate	
λ-Carrageenan	D-Galactose-2-sulfate	(1➔4)-β-
	D-Galactose-2,6-disulfate	
Konjac glucomannan	D-Glucose	(1➔4)-β-
	D-Mannose	
Xanthan gum	D-Glucose 2	(1➔4)-β-, main chain
	D-Mannose 2	(1➔3)-α-, (1➔4)-β-
	D-Glucuronic acid	(1➔2)-β-
	Pyruvic acid	
	Acetic acid	
Scleroglucan	D-Glucose	(1➔3)-β-, main chain (1➔6)-β-, side chain
Pullulan	D-Glucose	(1➔4)-α-, (1➔6)-α-
Dextran	D-Glucose	(1➔6)-α-, main linkage
Alginate	D-Mannuronic acid	(1➔4)-β-
	D-Guluronic acid	(1➔4)-α-
Pectin	D-Galacturonic acid	(1➔4)-α-
	Methanol*	
Gellan gum	D-Glucose 2	(1➔4)-α-, (1➔4)-β-
	D-Rhamnose 1	(1➔3)-α-
	D-Glucuronic acid 1	(1➔4)-β-
Locust bean gum	D-Mannose	(1➔4)-β-, main chain
	D-Galactose	(1➔6)-α-, side chain
Starch amylose	D-Glucose	(1➔4)-α-
Starch amylopectin	D-Glucose	(1➔4)-α- (1➔6)-α-, branching point

* A preparation containing 28-39% methyl ester was used

as the gels obtained by cooling heated aqueous suspensions but heat-induced gels of curdlan differ fom the neutralized gels.

Scleroglucan, xanthan gum, succinoglycan, λ-carrageenan, dextran, pullulan, and locust bean gum form viscous aqueous solutions. Starch granules swell and form sols when heated in aqueous suspensions.

Experimental Techniques

1. Low-molecular mass glucans.

Low-molecular mass (1→3)-β-D-glucans [18] having DPn 131 and 49, were obtained from curdlan by partial hydrolysis using 85% formic acid at 81-90°C (DPn 49) or at 76-83°C (DPn 131) for 30 min, followed by fractionation at Takeda Chemical Ind. Ltd.

Short-chain (1→4)-α-D-glucans (amylose) [16] having DPn 18, 57, 78, and 117 were prepared by debranching waxy corn amylopectin with *Pseudomonas* isoamylase followed by fractionation. The materials were kindly supplied by the Hayashibara Biochemical Institute in Okayama City.

2. Preparation for electron microscopy.

Samples of 0.05% or 0.1% neutralized or heated gels or viscous solutions were prepared for electron microscopy by negative staining or metal shadowing. Neutralized gels were obtained from alkaline solutions in 0.1 or 0.3 N sodium hydroxide by dialysis against water to remove the sodium hydroxide. Heated gels were obtained by heating aqueous solutions at the boiling point for 10 min and subsequently cooling them to 30°C.

Carbon film (~15 nm thick) formed by vacuum evaporation on freshly cleaved mica sheets was transfered on transmission electron microscopy (TEM) grids, dried in air, and annealed by heating at 100°C for 4 h. The carbon film was then subjected to ion-cleaning by glow discharge at low pressure to remove any oily material in order to prevent any artifactual assemblance of the matter under study. Samples (10 μl of 0.05% or 0.1% neutralized or heated gels or viscous solutions) were deposited on the carbon film, dried in air, and prepared for electron microscopy by negative staining or metal shadowing. A droplet (10 μl) of a 2% uranyl acetate solution, pH 4.4, was placed on the surface of the dried sample and kept for several minutes. The solution residue was removed by absorption using filter paper. The negatively stained sample was dried in air in a dust-free fume hood. For metal shadowing, the unstained preparation on the TEM grid was shadowed with platinum at an angle of 30°.

The negatively stained or shadowed preparations were examined in a Hitachi H-600FE electron microscope equipped with a field emission gun and operated at an accelerating voltage of 100 kV. Micrographs were taken at an original magnification of 50,000X.

3. Molecular association in curdlan.

As has already been mentioned, curdlan forms two types of gel [17, 18]). One type is formed by neutralization of its alkaline solutions or by heating aqueous suspensions at 60°C and subsequently cooling below 40°C. The other type is formed by heating aqueous suspensions above 80°C and subsequent cooling. When the gels had been frozen and subsequently thawed, the neutralized gels showed a considerably higher syneresis than the heat-induced gels [17, 38]. The two types of gel are formed by different mechanisms. The properties of the neutralized gels are similar to those of gels of agar and κ-carrageenan.

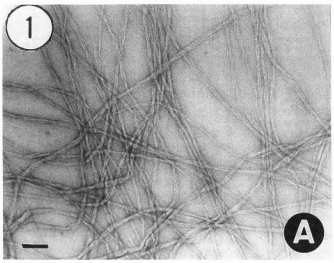
Molecular association in curdlan gels formed by neutralization of alkaline solutions using carbon dioxide was compared with molecular association taking place in gels obtained by heating aqueous suspensions at various temperatures [10]. Neutralized gels and gels set at 60°C were soluble in 0.01 N sodium hydroxide solutions whereas gels set at above 95°C were soluble in sodium hydroxide solutions only at concentrations higher than 1 N. The adsorption of Aniline Blue or Congo Red on the preparations decreased with the increase in the temperature of the heat treatment: the adsorption on a gel heated at 120°C for 4 h was about 30% of that on unheated and neutralized gel. Residual amounts of the dyes obtained by centrifugation of dyed curdlan samples were determined photometrically and the amounts of the dyes adsorbed were calculated. On treatment with 32% sulfuric acid for 30 days, 73% of the heated preparation (120°C for 4 h) was resistant to the treatment [18], whereas the neutralized gel was not resistant.

Electron microscopy studies of curdlan [10, 18, 22, 39] show the neutralized gels following dialysis against water from a 0.3 N sodium hydroxide solution (Figs. 1A and 1B) and from a 0.1 N sodium hydroxide solution (Fig. 1C). The neutralized gel from 0.1 N sodium hydroxide showed an irregular form of microfibrils having fibril units about 100 nm long, but a gel from 0.3 N sodium hydroxide produced clearer long microfibrils, 10-25 nm wide. The microfibrils were composed of several subunits 2-3 nm wide as shown by an arrow in Fig. 1B. Ogawa *et al.* [27] reported on the conformation of curdlan that it occurs as random coils in sodium hydroxide solutions at concentrations higher than 0.24 N but has an ordered conformation at lower sodium hydroxide concentrations.

The mass resulting from sonication of gels was also examined by electron microscopy: the material obtained by dialysis from a 0.3N sodium hydroxide solution is shown in Fig. 1D and the material obtained by neutralization of the alkaline solution using hydrochloric acid is presented in Fig. 1E. Elementary fibril units of microfibrils about 100 nm long and about 20 nm wide may be seen in the latter micrograph. The images in Fig. 1D do not appear as clear as in Fig. 1E. The fibril units were loosened by heating at 55°C and released many considerably thinner elementary fibril units 1-3 nm wide (Fig. 1F), but heating at 60°C caused these scattered elementary fibril units to form microfibrils similar to those shown in Fig. 1B. Single elementary fibrils may have a structure composed of several molecules as shown in a model proposed on the basis of x-ray diffraction studies [21].

Curdlan powder is solubilized at 55°C and forms gel on cooling after heating above 60°C [23]. The elementary fibril units (Fig. 1F) are connected with each other in axial direction to form considerably longer microfibrils. The microfibrils are probably composed of fibril units about 100 nm long. This finding is supported by observations of aqueous suspensions heated at 120°C (Fig. 2A-C), which contained pseudocrystalline forms about 100 nm long. Gels formed after heating at 95°C (Fig. 2D) were composed of a network of interconnected microfibrils and elementary fibril units. Treatment of this latter gel with zymolyase, which is a (1→3)-β-D-glucanase, caused the release of elementary fibril units (Fig. 2E) from the fibril units.

The formation of gels by neutral polysaccharides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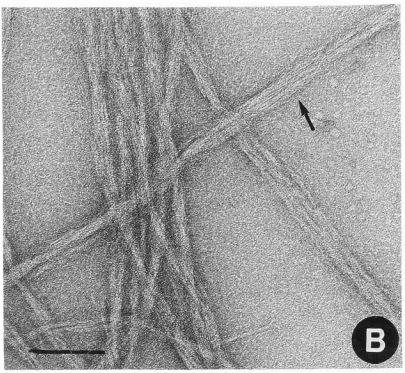
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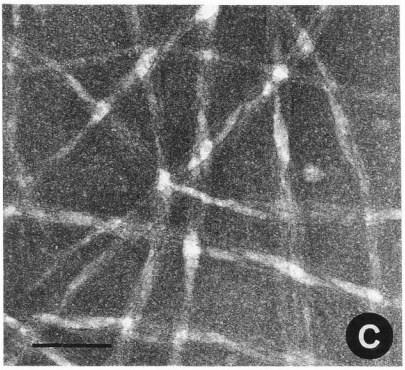
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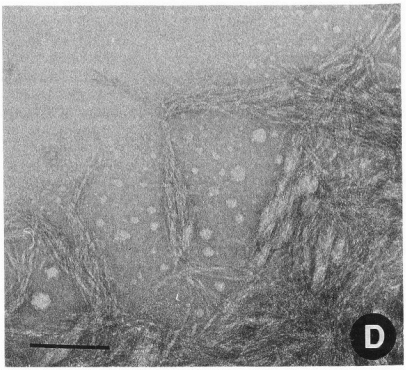
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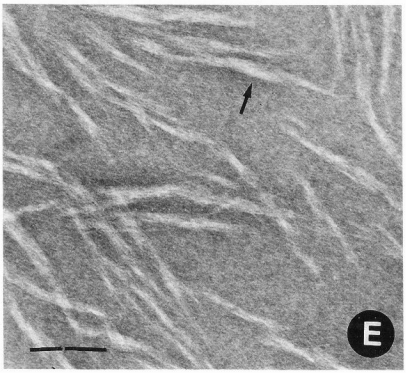
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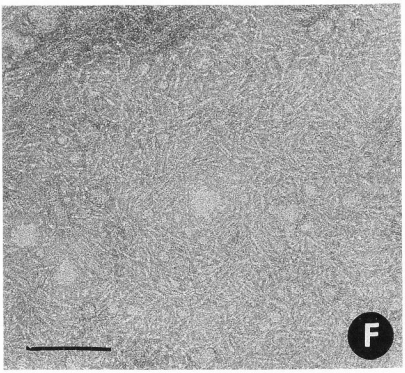
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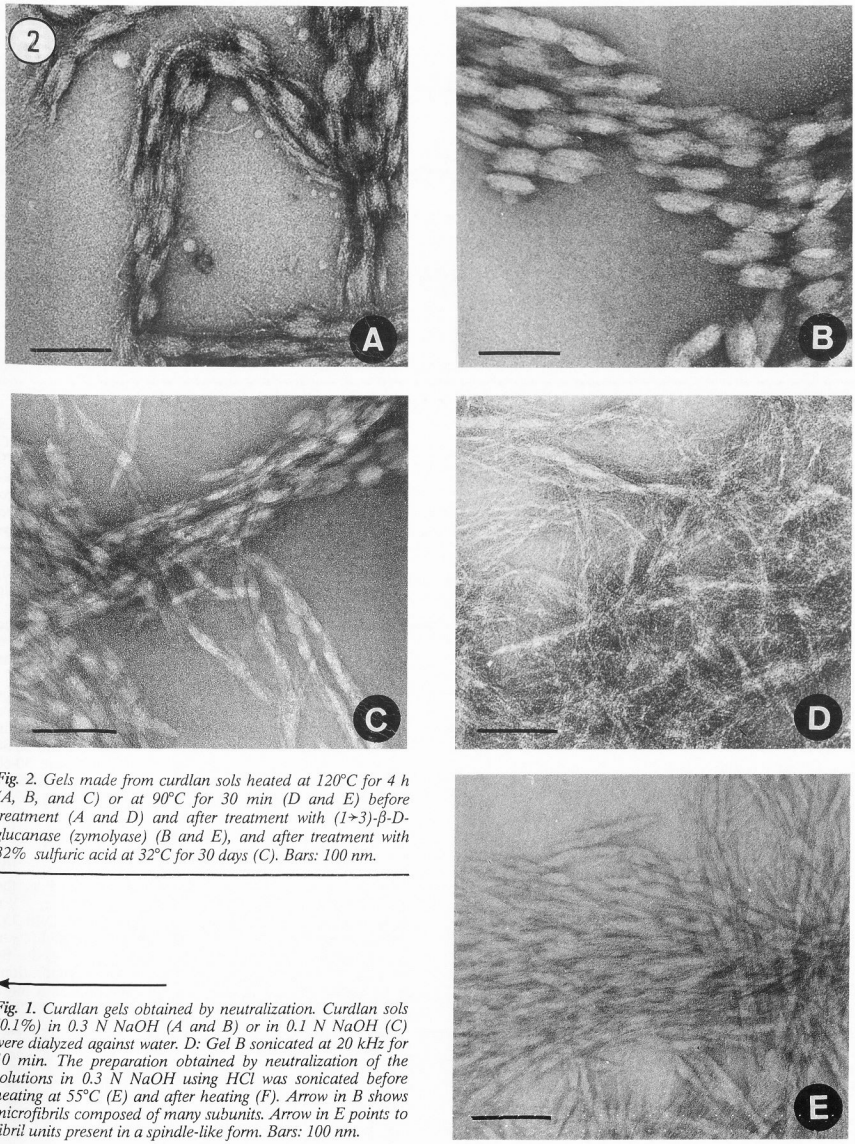


Fig. 2. Gels made from curdlan sols heated at 120°C for 4 h (A, B, and C) or at 90°C for 30 min (D and E) before treatment (A and D) and after treatment with (1→3)-β-D-glucanase (zymolyase) (B and E), and after treatment with 32% sulfuric acid at 32°C for 30 days (C). Bars: 100 nm.

Fig. 1. Curdlan gels obtained by neutralization. Curdlan sols (0.1%) in 0.3 N NaOH (A and B) or in 0.1 N NaOH (C) were dialyzed against water. D: Gel B sonicated at 20 kHz for 10 min. The preparation obtained by neutralization of the solutions in 0.3 N NaOH using HCl was sonicated before heating at 55°C (E) and after heating (F). Arrow in B shows microfibrils composed of many subunits. Arrow in E points to fibril units present in a spindle-like form. Bars: 100 nm.

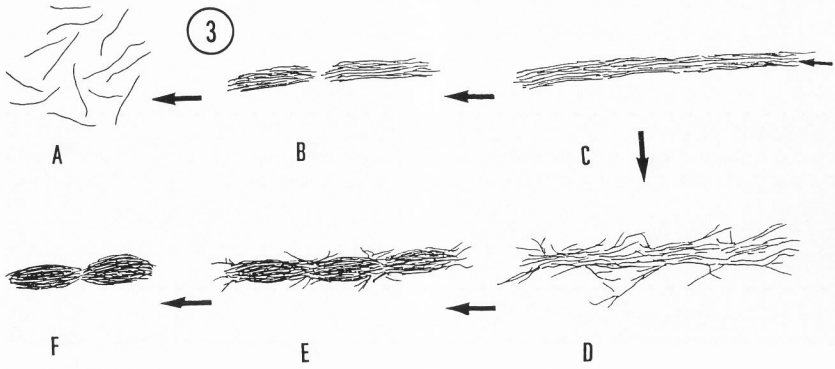


Fig. 3. Schematic view of changes in the forms of curdlan. A: elementary fibril units, B: fibril units, C: microfibril (small arrow points to microfibril subunits), D: microfibril net-like structure, E: pseudocrystalline form consisting of elementary fibril units and microfibrils, F: pseudocrystalline form. C→B (sonication), B→A (heating at 55°C), C→D (heating at 95°C), D→E (heating at 120°C), E→F (treatment with (1→3)-β-D-glucanase or 32% sulfuric acid at 32°C.

Fig. 4. Degraded curdlan of $\overline{DP}n$ 49 (A, C) and $\overline{DP}n$ 131 (B and D) prior to heating (A and B) and after heating at 120°C for 4 h (C and D). Crystalline forms in the $\overline{DP}n$ 49 preparation examined by negative staining (E) and metal-shadowing (F). Small arrows in C and D point to pseudocrystalline forms; large arrows in C, E, and F point to crystalline forms. Bars: 100 nm. ⟶

such as curdlan is an interesting phenomenon. From experiments on synergism of locust bean gum and xanthan gum (as will be discussed in Section 8), Dea and Morris [3] concluded that both associating and nonassociating molecular regions are essential for gel formation. Electron-dense structures were noticed in gels made following heating at 120°C (Fig. 2A). The microfibrils in this gel are considerably wider than in the neutralized gel shown in Fig. 1B. Microfibrillar units are released and then seen to reassociate with each other to form pseudocrystals about 100 nm long. In curdlan gel formed following heating at 120°C for 4 h, the parts that were resistant to zymolyase [39] (Fig. 2B) and acid [18] (Fig. 2C) treatments contained similar spindle-like pseudocrystalline forms. X-Ray diffraction patterns of the gels treated with zymolyase or 32% sulfuric acid were almost the same [18, 39]. Preparations having $\overline{DP}n$ <200 were unable to form gels. Our proposal to view curdlan microfibrils as composed of fibril units which are further composed of elementary fibril units is illustrated by Fig. 3.

Fulton and Atkins [5] based their hypothesis on the gelling mechanism on x-ray diffraction and infrared spectroscopy. Their hypothesis differs from that by Saito *et al.* [34] and Okuyama *et al.* [28] as well as from ours [40], as will be shown in Section 8. Fulton and Atkins [5] emphasized the importance of the triple helical molecules bound by hydrogen bonds to the interstitial water of crystallization and the resulting formation of micellar domains. Saito *et al.* [34], Okuyama *et al.* [28], and Kasai, and Harada [21] postulated that single and triple helical structures participate in the formation of neutralized and heat-induced gels, respectively.

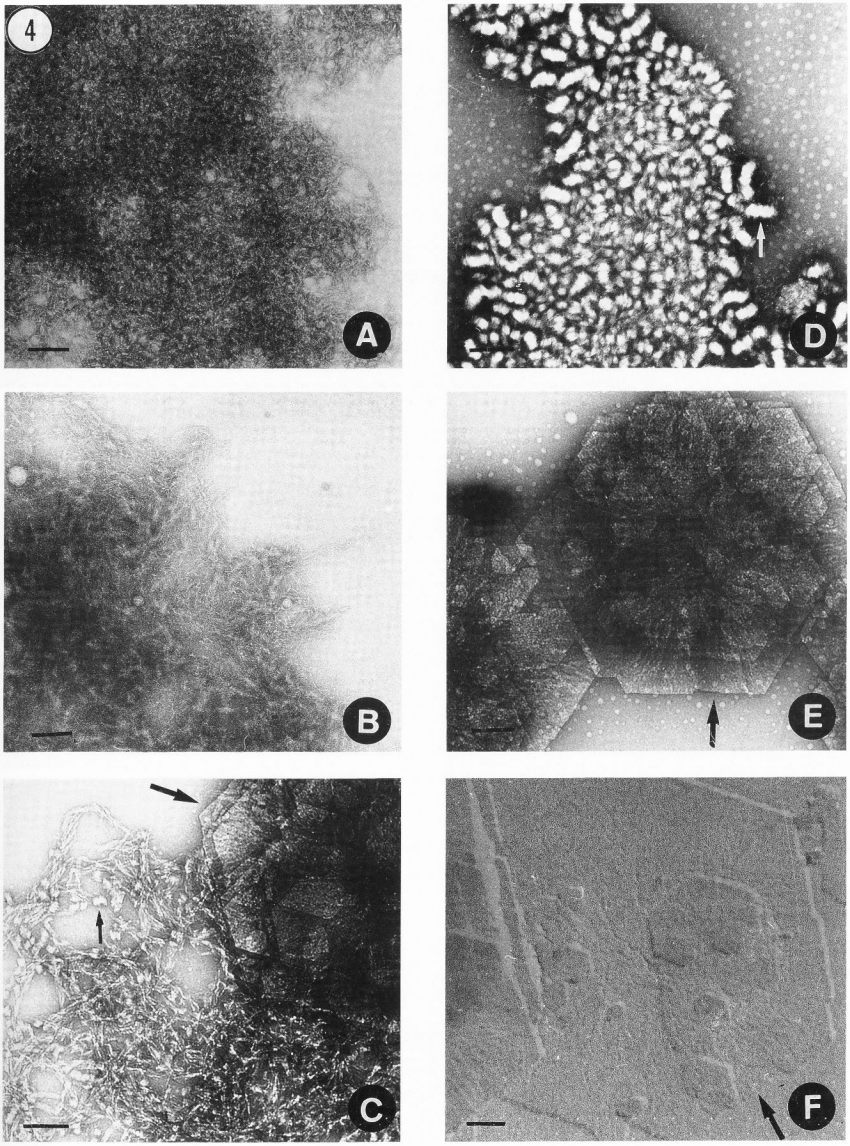
Unheated preparations having $\overline{DP}n$ 49 or 131 produced shorter and thinner microfibrils (Figs. 4A and B) [10] than the original curdlan of $\overline{DP}n$ 450. When heated in an aqueous suspension at 120°C [10], the preparation of $\overline{DP}n$ 49 developed both pseudocrystalline forms and hexagonal lamellar crystals (Figs. 4C and F), whereas preparations of $\overline{DP}n$ 131 contained only pseudocrystalline forms (Fig. 4D).

Metal-shadowing revealed that the thickness of the crystals was about 8.5 nm in heat-induced gels of the $\overline{DP}n$ 49 preparations (Fig. 4F). The pseudocrystalline forms in the $\overline{DP}n$ 49 and 131 preparations were 10-15 nm and 20-30 nm wide, respectively; their respective lengths were 30-60 nm and 80-100 nm. There is a possibility that the molecules were aligned at right angles to the axes of the strands.

As was mentioned earlier, the fibril units of the original curdlan are about 100 nm long (Fig. 1E). Considering that the lengths of the microfibrils are reflecting the lengths of the molecules, the values of 100 nm, 20-30 nm, and 10-15 nm may be regarded as the lengths of the fibrous units of the original curdlan ($\overline{DP}n$ 450) and the degraded curdlan preparations of $\overline{DP}n$ 131 and 49, respectively. Here, we point out that the subunits in the microfibrils are oriented parallel to the strands. By heating the original curdlan or the degraded preparations at higher temperatures, the molecules associate in a more compact manner and produce crystals or pseudocrystalline structures.

4. Molecular association and gel-forming ability of polysaccharides used in foods [9, 19].

Polysaccharides insoluble in cold water such as agar and κ-carrageenan form gels when their aqueous solutions obtained by heating are cooled. Curdlan alone and konjac glucomannan in the presence of calcium hydroxide form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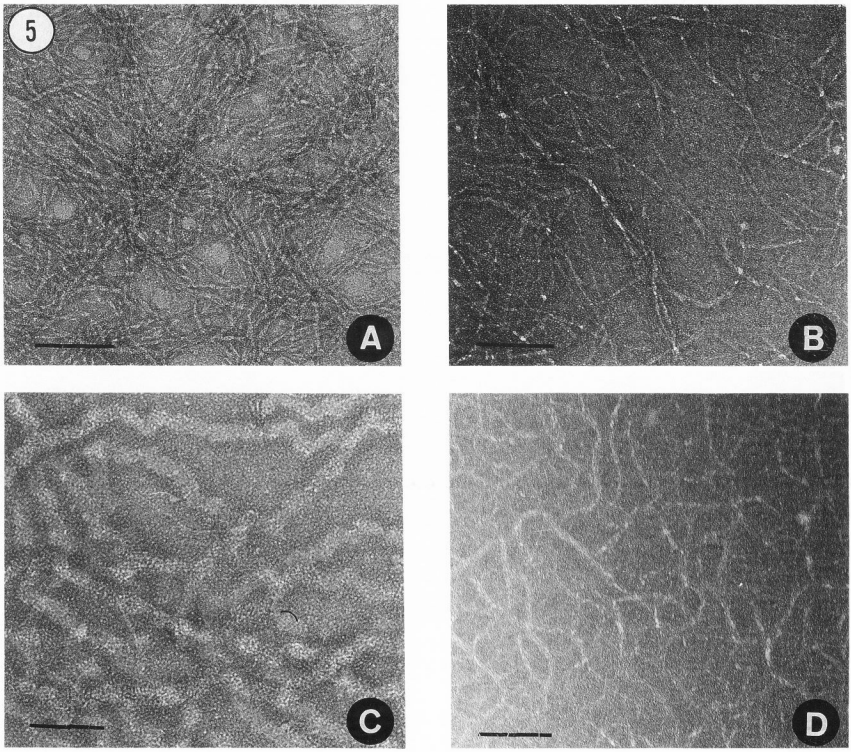
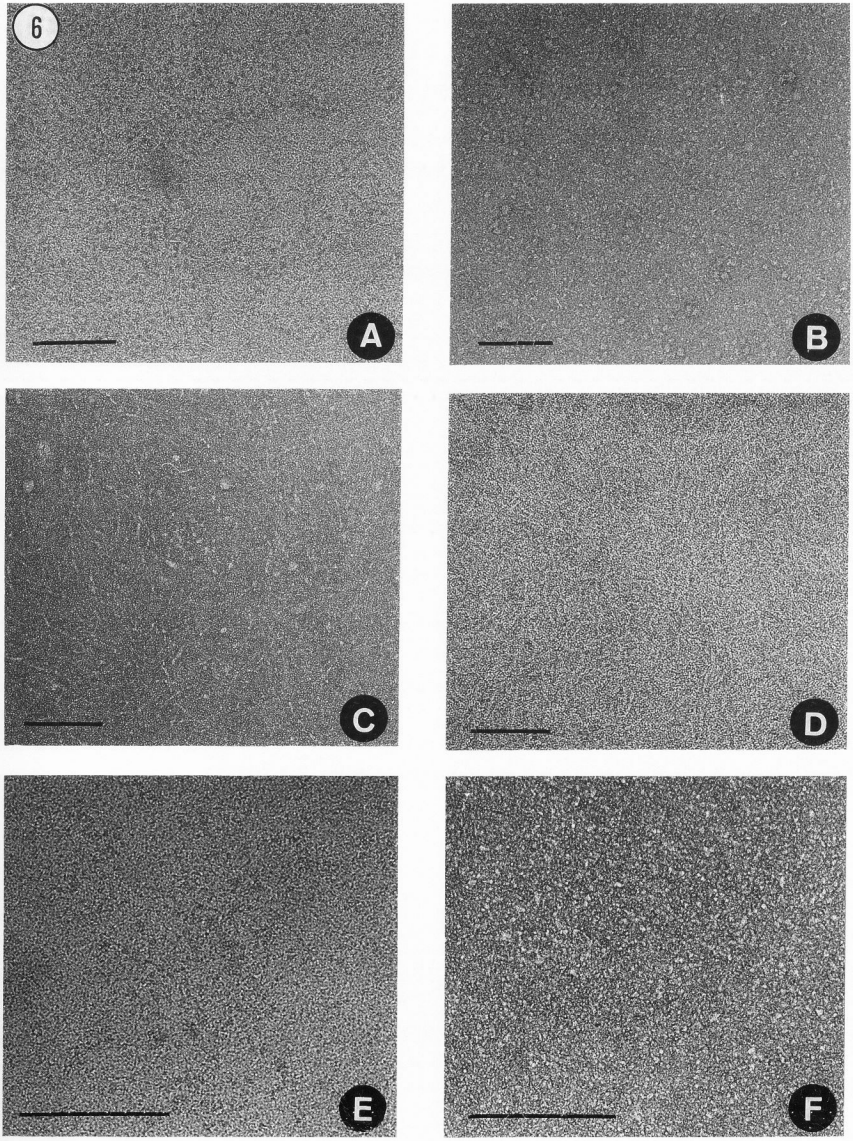


Fig. 5. Neutralized gels of agar (A), κ-carrageenan (B), konjac glucomannan (C), and ι-carrageenan (D). Bars: 100 nm.

Fig. 6. Mucous polysaccharides (sols): λ-carrageenan (A), scleroglucan (B), succinoglycan (C), xanthan gum (D), pullulan (E), and dextran (F). Bars: 100 nm. →

gels above 80°C. Agar, κ-carrageenan, konjac glucomannan, and ι-carrageenan form gels when their alkaline solutions are neutralized either by dialysis or by using carbon dioxide. Other acids are not suitable because stirring required to distribute the acid immediately breaks the originating gels. Chanzey and Vuong [1] obtained electron micrographs showing microfibrils in gels of curdlan, konjac glucomannan, and carrageenans. Water-soluble and mucous polysaccharides such as λ-carrageenan, scleroglucan, succinoglycan, xanthan gum, pullulan, and dextran do not form gels.

Electron micrographs show [19] that neutralized gels of agar, κ-carrageenan, and konjac glucomannan are composed of interconnected long microfibrils, each 5-25 nm wide; the microfibrils of ι-carrageenan are shorter (Fig. 5). Viscous solutions are composed of considerably shorter microfibrils about 1-2 nm wide (Fig. 6). With the exception of curdlan, the structures of gels formed by cooling heated sols are similar to gels formed by neutralization. These findings indicate that long and/or wide microfibrils are required for the formation of a gel. Holzwarth and Prestridge [14] and Stokke *et al.* [37] studied the conformation of xanthan gum by electron microscopy and observed single- and double-stranded fibrous forms (Fig. 7). The latter authors showed that the polymer exists as a double-stranded fibre 4 nm wide and a single-stranded fibre 2 nm wide. These authors used the metal-shadowing method, in which the lateral point-to-point resolution is known to be about 3 to 5 nm. The images (Fig. 7) are clearer than our images obtained by the negative staining technique. One reason for this may be the effect of the 2% uranyl acetate solution on the sample in the negative staining procedure.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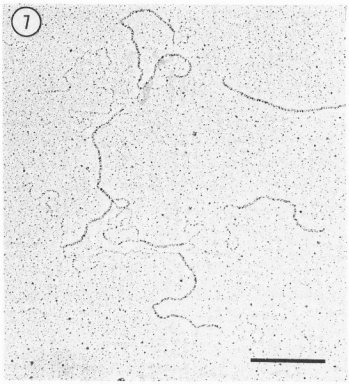
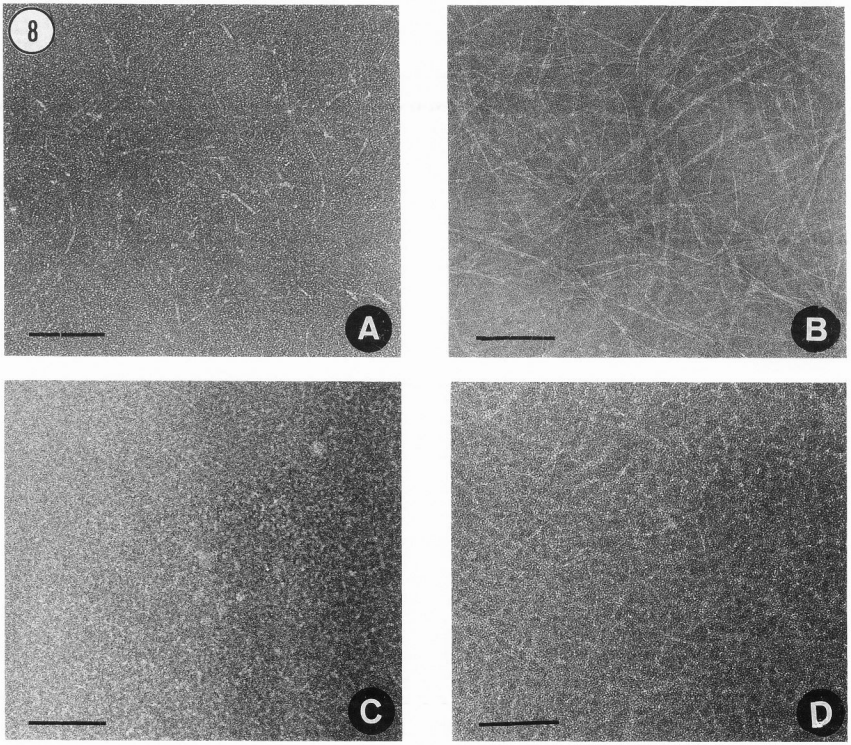


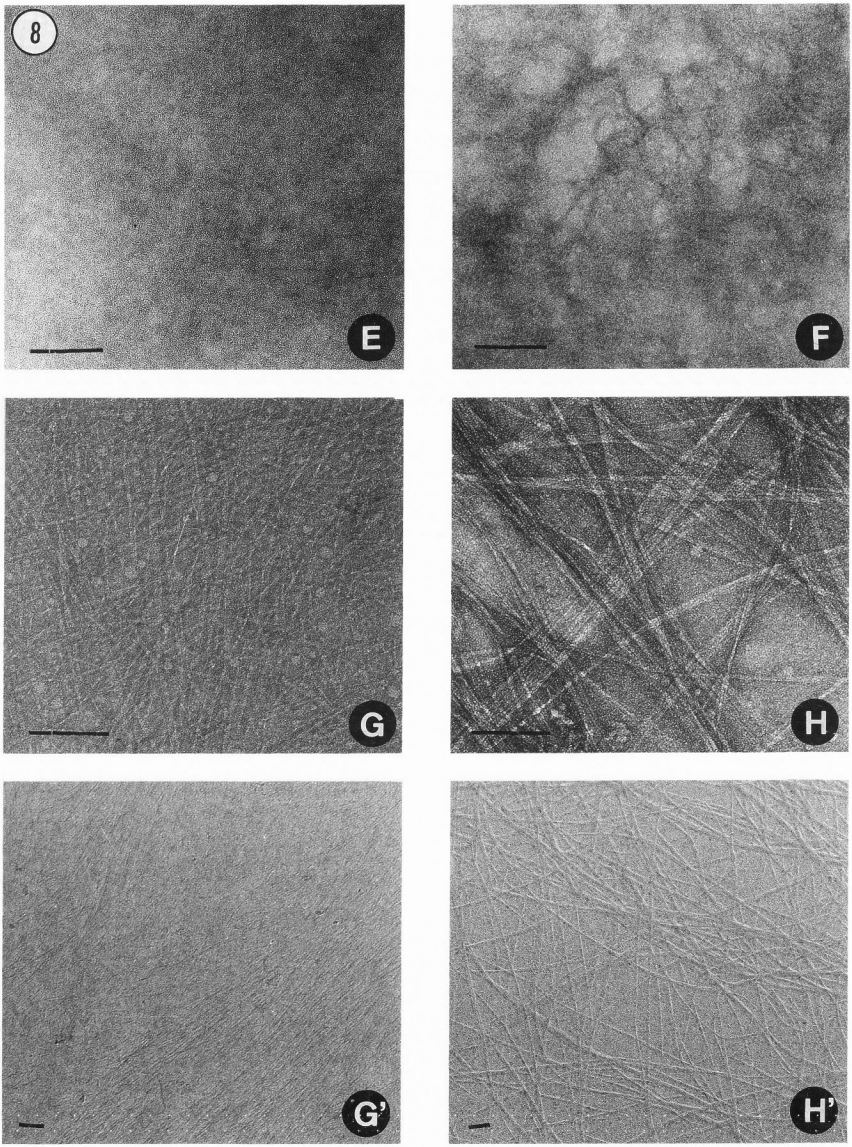
Fig. 7. Xanthan gum (metal-shadowing). Bar: 100 nm. (Courtesy of B. T. Stokke et al.) [37].

5. Molecular association and gel formation in the presence of certain cations [20].

The firmness of κ-carrageenan gels is known to be increased by the presence of potassium ions [13] whereas aqueous solutions or suspensions of alginate [29], gellan gum [6], and low-methoxyl pectin [26] form gels in the presence of calcium ions.

Fig. 8. Comparison of the images of κ-carrageenan (A and B), sodium alginate (C and D), low-methoxyl pectin (E and F), and gellan gum (G, G', H, and H') obtained by negative staining (A-H) and by metal-shadowing (G' and H'). The polysaccharides are shown in the absence of cations (A, C, E, G, and G') and in the presence of potassium (B) or calcium ions (D, F, H, and H'). Bars: 100 nm.

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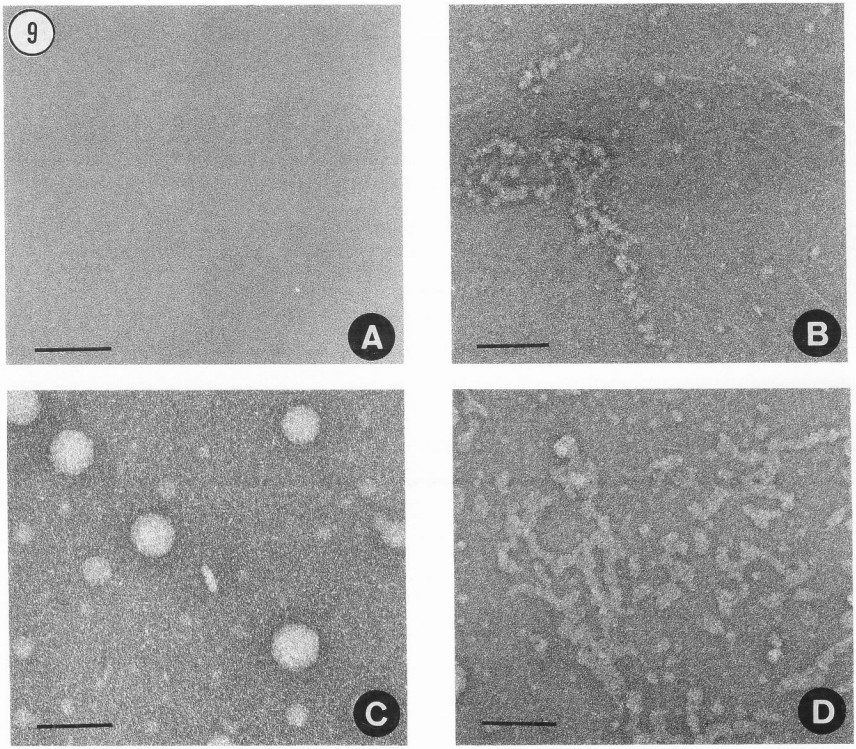
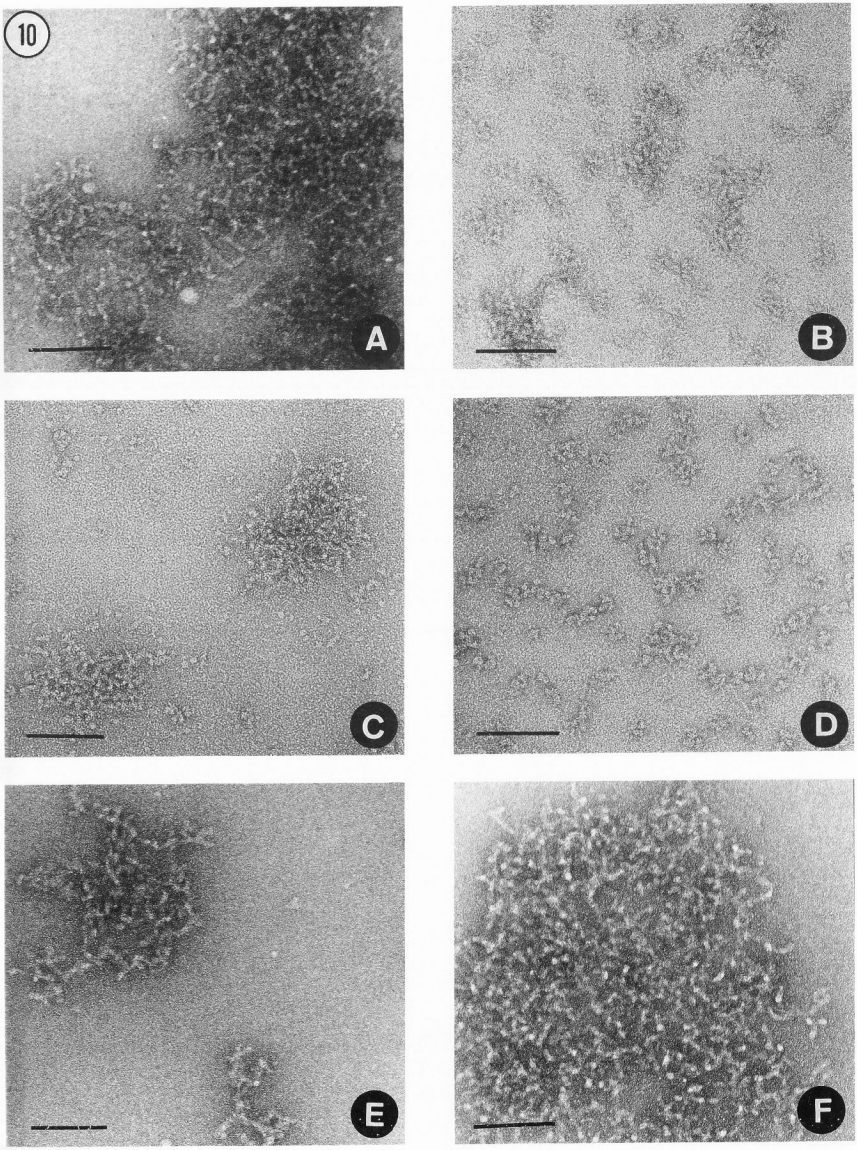


Fig. 9. A locust bean gum sol (A) and gels composed of locust bean gum and κ-carrageenan (B), and locust bean gum and xanthan gum (C and D). C: Gel obtained by cooling a heated mixture, D: gel obtained by dialysis of a mixture in an alkaline solution. Bars: 100 nm.

Fig. 10. Gelatinized starch preparations obtained by dialysis of solutions in 0.4 N NaOH against water. The starches were prepared from waxy corn (A), corn (B), wheat (C), cassava (D), sweet potato (E), and potato (F). Bars: 100 nm. ⟶

In our experiments, longer and/or wider microfibrils of the aforementioned polymers were observed in the presence of specific cations (Figs. 8B, D, F, H, and H') than in their absence (Figs. 8A, C, E, G, and G'). It is possible, however, that the ions of uranyl acetate used for negative staining may act as ions competing with the ions promoting gelation and may have induced changes in the structure of the polymers as suggested by Stokke *et al.* [37]. We stained the preparations only after they had been dried on the carbon film-coated grid and then the residual uranyl acetate solution was removed. Thus, we reduced the possibility of a competitive action between uranyl acetate and the specific cations. In order to confirm the effect of specific cations on structural patterns of the polysaccharides, gellan gum was examined by the metal-shadowing technique [14], which is known not to involve the use of competitive ions. Both methods produced similar results (Figs. 8G, G', H, and H'). Recently, Hermansson [13] used the mica sandwich technique to show that the aggregation of κ-carrageenan molecules depends on the potassium ion concentration. In this technique, a thin layer of the solution under study is placed between two freshly cleaved mica surfaces. The sample is rapidly frozen and then freeze-dried.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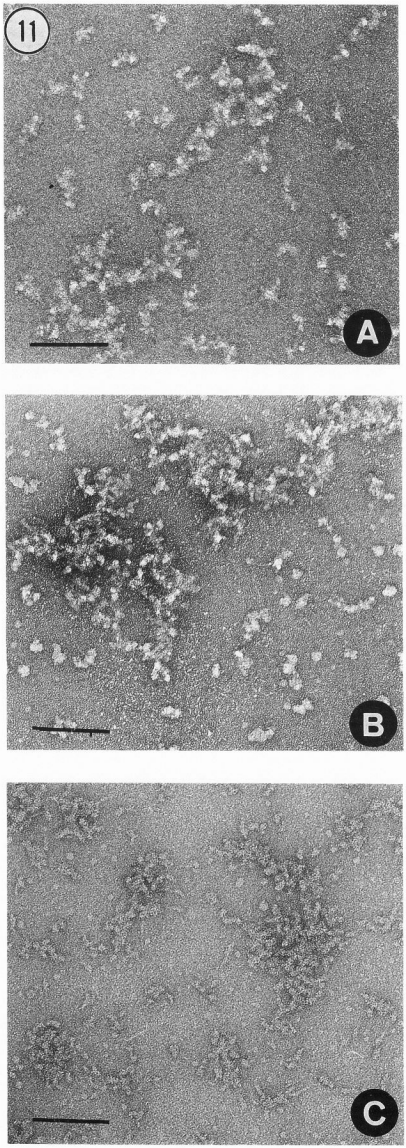

6. Association of molecules of two kinds of polysaccharides having similar partial structures.

The polysaccharides dealt with in this section have similar backbones. Locust bean gum contains (1→4)-β-D-linked mannose, κ-carrageenan contains (1→4)-β-D-linked galactose, and xanthan gum contains (1→4)-β-D-linked glucose residues. Unsubstituted regions of the glycosidic chains are implicated in junction formation [4].

The firmness of κ-carrageenan gels made in the presence of potassium ions is known to be increased by the addition of locust bean gum which is incapable of forming a gel alone [29]. On the other hand, locust bean gum is also known to form gels in the presence of polysaccharides which do not form gels alone, such as xanthan gum [4]. Micrographs of locust bean gum in the absence and presence of κ-carrageenan or xanthan gum are presented in Figs. 9A-D. Interconnected globular or rodlike structures were observed in preparations containing both different polysaccharides.

7. Amylose and amylopectin in gelatinized starch [15, 16].

Structures of granular waxy corn starch were studied by TEM by Yamaguchi *et al.* [41]. These authors observed lamellar forms as linear regions of amylopectin and highly elongated irregular particles approximately 10 nm in diameter and several tens of nm long in a gel obtained from a solution of amylopectin in dimethyl sulfoxide. There are no reports on TEM studies of the ultrastructure of amylose. Our micrographs of gelatinized starch are presented in Figs. 10A-F. The preparations were obtained by cooling aqueous suspensions of starch heated to 95°C for 10 min or by dialyzing alkaline solutions of starch in 0.4 N sodium hydroxide against water. The latter preparations produced clearer images by electron microscopy than the heated suspensions but the reasons for this difference are not known.

No significant differences were observed between micrographs of waxy corn amylopectin and other starches composed of amylose and amylopectin. Amylopectin molecules have many linear parts containing (1→4)-α-D-glucosidic linkages and (1→6)-α-D-glucosidic linkages at branches whereas amylose consists of long straight chains connected with each other entirely by the former linkages. The average chain length of glucose in amylopectin is between 18 and 24 units, whereas the chains in wheat and potato amyloses are 1,000-2,000 units and 4,000 units long, respectively [24]. The cluster model is accepted for amylopectin in the starch granules [24]. Amylose chain molecules may, therefore, combine with amylopectin molecules to produce various forms.

We also examined amylose preparations obtained from corn, cassava, and potatoes (Figs. 11A, B, and C, respectively). Rod-shaped or irregular forms and sometimes branched forms about 10 nm wide were observed. Low-molecular mass amylose preparations having DPn of 18, 57, 78, and 117 were also examined (Figs. 12A-D). The DPn 18 preparations had the A-type crystalline form but the other preparations showed neither the A- nor the B-type crystals [16]. A and B patterns are genuine crystalline modifications. The A pattern is usually present in cereal starches whereas the B pattern is found in potato starch. The structural element of the B pattern is a double helix packed in an antiparallel, hexagonal mode. The A pattern is similar,

Fig. 11. Amylose preparations obtained by dialysis of solutions in 0.4 N NaOH. The amyloses were prepared from corn (A), cassava (B), and potato (C) starches. Bars: 100 nm.

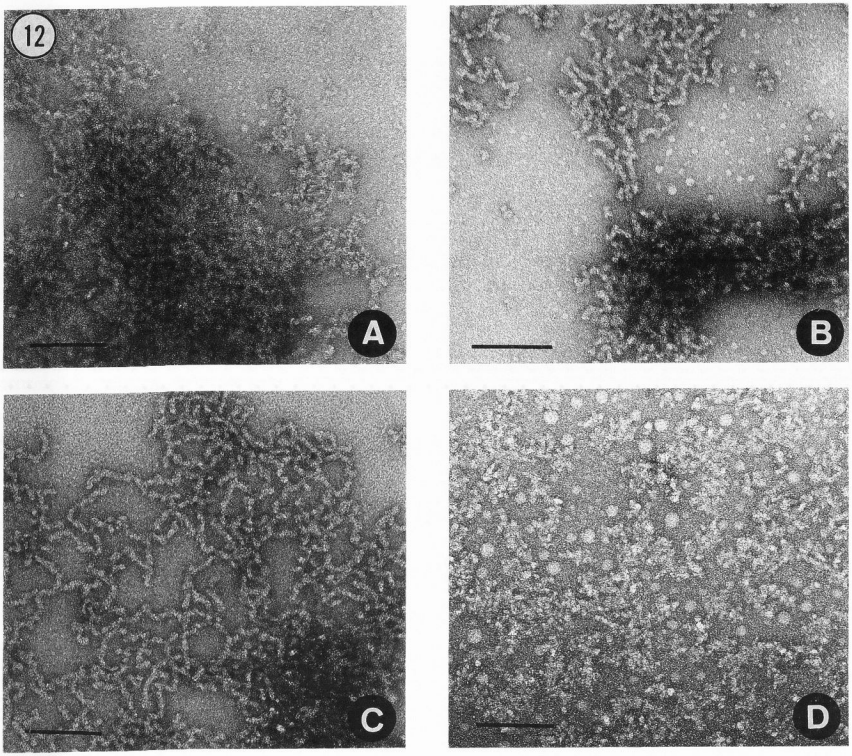

Fig. 12. Low-molecular amylose preparation obtained by dialysis of 0.4 N NaOH solutions against water. A: $\overline{DPn}$ 18, B: $\overline{DPn}$ 57, C: $\overline{DPn}$ 78, D: $\overline{DPn}$ 117. Bars: 100 nm.

except that the central channel is occupied by another double helix. The $\overline{DPn}$ 57 and 78 preparations showed clear long branched microfibrils about 10 nm wide. In the micrographs, streaks may be seen to run at an angle to the axes of the microfibrils. The microfibrils in amylose and amylopectin are similar in appearance and, thus, electron microscopy cannot be used to make the distinction between them.

8. Additional aspects.

Molecular aggregations observed in these studies are the consequence of double-, triple-, or multiple-strand helices but also involve hydrogen bonding and/or hydrophobic reactions. The micrographs used in this study to illustrate the associations were taken under conditions in which the gels were sufficiently diluted to be seen directly by TEM.

Associations leading to cooperative cross-linkages make it possible for polysaccharides such as alginate, low-methoxyl pectin, and gellan gum to form gels [29]. Here, divalent ions such as calcium promote the association of the polysaccharide molecules. Molecules of κ-carrageenan have been known to associate by ion-selective salt bridges formed by potassium ions. These ionotropic gels probably originate by a mechanism in which the gel-inducing ions form bridges between the polymer chains only at specific points distributed along the molecular chain. This view is supported by the x-ray diffraction patterns of gellan gum in which the only differences were found between the ion-induced gel and the sol from which the ions were absent [20].

Gelation of neutral polysaccharides such as curdlan and amylose is both interesting and important because the ions, which supposedly promote gelation, are not present in the gels. Authors associated with Sarko [35, 36] and Marchessault [3, 25] reported that concerning crystalline

packing, the structure of curdlan gel resembles an amylose sol except that the former polysaccharide has a triple-stranded helix whereas the latter polysaccharide has a double-stranded helix. In contrast, Saito's group [31-34] demonstrated on the basis of high-resolution conventional and solid-state ^{13}C NMR studies that the triple-stranded helix portion represents only about 10% of the curdlan powder and that this portion is involved in the development of cross-links with flexible single-stranded helical chains. The proportion of the triple-stranded helix is increased up to about 50% by heating the gel to 150°C and then increased again, to 100%, by slowly cooling the gel.

Kasai's group [21, 40] concluded that there is a coexistence of the single and triple helices in non-heated curdlan specimens as detected by x-ray diffraction analysis. Okuyama *et al.* [28] detected the existence of the single helix in neutralized curdlan gels having <95% moisture content, whereas a single-chain structure was found in the dry state. Here we wish to notice the report by Chanzy *et al.* [2] on an electron diffraction study of starch because these authors examined high-moisture (95%) samples using a special cryostage technique. Both Kasai's and Saito's groups concluded independently that the single-stranded helices in curdlan are converted into triple-stranded helices at a higher temperature. Images of curdlan obtained by these authors at temperatures below 60°C differ considerably from those obtained by Marchessault's [3, 25] and Atkins' [5] groups and also from the well-known gelation mechanism of Rees [29, 30], established by x-ray diffraction studies. Based on the views of the Saito's, Kasai's, and Harada's groups, the single-stranded helix seems to participate largely in the formation of neutralized curdlan gels whereas the triple-stranded helix is involved in the formation of gels at temperatures above 120°C. Additional association of the single-stranded and triple-stranded helices to form the microfibrils may arise from hydrogen bonding in the neutralized gels and from hydrophobic interactions accompanying dehydration in the gels set at high temperatures. These processes are well illustrated by our micrographs. However, the participation of double- and triple-stranded helices is not the only mechanism by which gels are formed. Paramylon, *e.g.,* cannot be gelled [3] although its entire molecular chain is in the form of a triple-stranded helix. Several branched (1→3)-β-D-glucans isolated from fungi consist of bundles of triple-stranded helical chains, yet the gels, which they form, are weak [34].

Our experiments have shown that the molecules in original curdlan are aligned parallel to the axes of the microfibrils but the molecules in degraded curdlan preparations having $\overline{DP}n$ 131 and 49 seem to be arranged at a right angle to the axes of the pseudocrystals; molecules in native amylose sols seem to form rod-shaped or irregular structures whereas the molecules in short-chain amylose preparations having $\overline{DP}n$ of 57 and 78 show an arrangement similar to that in degraded curdlan except that they form very long branched strands. Thus, the molecules of short-chained polymers apparently associate with each other sideways to the chains. There are differences in the molecular associations between native curdlan and amylose: in curdlan, the end groups of one molecule associate with the end groups of another molecule but this kind of association is not possible in native amylose. We are offering the following explanation: the fiber repeating period in curdlan is 2.26 nm long [21] whereas the repeating unit in amyloses A and B is only 1.05 nm long [34]. The curdlan fiber is straight and, thus, may become more easily positioned parallel to the axis of other molecules and form long microfibrils.

Acknowledgments

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Discussion with Reviewers

M. Axelos: The values of data on x-ray diffraction in gels, to which there are several references in the text, may be controversial because they concern only solid or very concentrated samples but may not be applicable to low-solids gels.
Authors: We think that it would not be so strange that the results of x-ray diffraction studies seem to be controversial. The different results may be attributed to experimental conditions of specimen preparation. A very similar structure has been proposed for specimens dried after heat treatment at higher temperatures: the triple-stranded helix has been reported.

By transmission electron microscopy we can usually examine suprastructure of curdlan in the dry state. We can obtain information on the microfibrils, fibril units, and elementary fibril units but not on molecular structure. We have to use the electron diffraction method in order to examine further structures in the elementary fibril unit at the molecular level. The use of cryostage or other special technique in electron micrography may be useful for this purpose.

M. Axelos: In view of using uranyl acetate for negative staining of the polysaccharides, don't you anticipate any interaction between this salt and other polyelectrolytes?
Authors: We used negative staining after drying the preparations deposited on the carbon film. Sorry for amending this description only in the revised manuscript. Clear micrographs were not obtained from samples of acidic polysaccharides in suspensions or solutions mixed with a small amount of 2% uranyl acetate on a grid covered with carbon film. We have not encountered interactions between the salt and other polyelectrolytes.