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Cholesteric-like crystal analogs in glucuronoxylan-rich cell wall composites: experimental approach of acellular re-assembly from native cellulosic suspension

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Summary. Many plant cell walls are constructed according to a helicoidal pattern that is analog to a cholesteric liquid crystal order. This raises the question whether the wall assembly passes through a true but temporary liquid crystal state. The paper focuses on experiments performed from aqueous suspensions of extracted quince slime, i.e., a cellulose/glucuronoxylan wall composite that presents a helicoidal order when observed in situ, within the enlarged periplasm of the seed epidermal cells. Experiments carried out in acellular conditions showed that a spontaneous reassociation into a helicoidal order can be obtained from totally dispersed suspensions. The ultrastructural aspect of the reassembled mucilage suspension was different according to the resin used (LR White or nanoplast, a water-soluble melamin resin). It was always typically polydomain, and when an order was visible it was cholesteric-like and similar to the in situ native organization. Transition states with many imperfections expressed the difficulty of the system to reassemble in the absence of constraining surfaces. The possible intervention of glucuronoxylan (GX) in the ordered assembly of the microfibrils was checked by: (1) progressive extraction of GX by trifluoroacetic acid (TFA). The extraction was associated to a control of the fraction by analysis of uronic acid contents and observation at the electron microscope level. Extraction of GX provoked the formation of a flocculent mass, the flocculation being more intense when the TFA was more concentrated; (2) progressive change of pH in order to analyze the influence of pH on flocculation. Low pH (ca. pH 3) led also to a flocculation of the suspension, but the floc was reversibly lost after dialysis against distilled water. The results indicate the antifloc role of the GX due to the anionic charges carried by the side-chains. However, the function of GX as helper twisting agent in the cholesteric-like reassembly must not be ruled out.

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Introduction

In the last few years, literature has emphasized the fact that in a great number of cellulosic cell walls, the wall design is basically a helicoidal pattern that is analogous to a cholesteric order (Vian et al. 1986; Roland et al. 1987, 1989; Emons 1988; Vian and Reis 1991; Satiat-Jeunemaitre 1992; Neville 1993; Wolters-Arts et al. 1993). The plant cell wall is a composite made of cellulose microfibrils associated with matrix components, among which the xylan family is the most represented. In a recent review, we have focused on cellulose/glucuronoxylan-based cell walls that present a helicoidal structural order (Reis et al. 1994).

The morphogenesis of cell wall is a complex multistep process that has given rise to an abundant literature. The question of polymer synthesis and guidance by the cell machinery (synthetases, cytoskeleton) is out of the scope of the present paper (see Reis et al. 1994 for recent discussion). We have chosen to focus on the events that occur when the wall polymers meet outside the plasma membrane. In particular, the analogy with liquid crystal leads to take into account possible spontaneous association processes. Indeed, liquid crystals are formed by self-assembly of compatible molecules. Concerning the cell wall, the question is whether the assembly passes through a true liquid crystal state, even

if a temporary one (Bouligand 1978, Jarvis 1992). To answer the question, several approaches were possible. One approach consisted in a careful analysis of various biological models constructed with helicoidal cell walls, in order to find structural defects, i.e., diagnostic features characteristic of liquid crystal organization. Hard tissues provide models that are favourable for that approach. They correspond to stony tissues and related structures. Hard models are permanent constructions and ensure a role of support and protection. Stone cells and endocarps are characterized by thick and ordered secondary walls showing a cholesteric-like pattern (helicoidal structure). Typical defects emerging in the stacks of microfibrils (disclinations, distortions, saddle-like figures) are diagnostic of an actual liquid crystal behaviour under mechanical constraints (Reis et al. 1992, 1994; Roland et al. 1993).

Another approach is to attempt to perform cholesteric reconstructions from native polymers under acellular conditions. For such experimentation soft models appear favourable. They correspond to cellulosic mucilages. Soft models are never consolidated, and their behaviour is a complete dispersion. Quince mucilage presents a typical helicoidal organization which is relevant to a true cholesteric liquid crystal state (Abeysekera and Willison 1988, 1990). It is known as a cellulose/glucuronoxylan composite (Mühlethaler 1950, Franke and Ermen 1969, Schnepf and Deichgräber 1983, Lindberg et al. 1990). A preliminary study showed that it is possible to obtain spontaneous ordered assemblies from aqueous suspensions of extracted quince slime (Reis et al. 1991). The present paper confirms these data and revaluates the experiments with a special attention on the role of glucuronoxylans (GX) in the cholesteric assembly.

Materials and methods

Material

Mature seeds were selected from fresh fruits of quince (*Cydonia oblonga* L.) collected directly on trees or bought on the market.

Extraction of the mucilage and in vitro re-assembly

Mature seeds were soaked during 2 to 3 days in distilled water at 4 °C and filtered on miracloth. The viscous suspension was centrifuged at 40,000 **g** for 11/2 h to get rid of impurities. The clear and viscous supernatant corresponded to the cellulosic suspension. The cellulosic suspension was concentrated by ultracentrifugation at 240,000 **g** for 17 to 24 h at 4 °C. The pellet was subsequently gently dehydrated at 4 °C for 2–3 days, giving rise to a viscous drop. The cellulosic suspension and the viscous drop were examined with a light microscope in bright field and with crossed polarizers.

LR White embedding

Mature seeds were fixed with 2% glutaraldehyde in 0.1 M cacodylate buffer for 2 h, then washed in cacodylate buffer. After dehydration in an ethanol series, the samples were embedded in LR White methacrylate.

Viscous drops of concentrated mucilage were directly dehydrated by pure ethanol and embedded in LR White resin.

Nanoplast embedding

Viscous drops were embedded in hydrosoluble melamin resin (nanoplast) according to Bachhuber and Frösch (1983). The specimen was directly put and oriented in flat molds filled with the nanoplast resin (10 g MME 7002, hexamethylol-melamine methyl ether, and 0.20 g of catalyst B 52). The molds were placed 24 h at room temperature, 24 h at temperature in a desiccator, and then, the desiccator was placed 48 h in an oven at 40 °C. The molds were removed from the desiccator and left to harden for 24 to 48 h at 60 °C.

Cytochemical characterization

For visualization of vic-glycol groups of polysaccharides, the ultrasections were stained by PATAg test (periodic acid-thiocarbohydrazide-silver proteinate).

Characterization of the carboxylic groups of the xylans were made on ultrathin sections. The sections were floated overnight at room temperature on 10 nm cationic gold particles conjugated to poly-L-lysine (Biocell) (Skutelsky and Roth 1986), diluted 1/50 in 0.05 M phosphate buffer, pH 5, and contrasted with 1% uranyl acetate.

Negative staining

Cellulose suspension was spread on Formvar-coated grids that were glow-discharged just before use. Grids were negatively stained using 1% uranyl acetate.

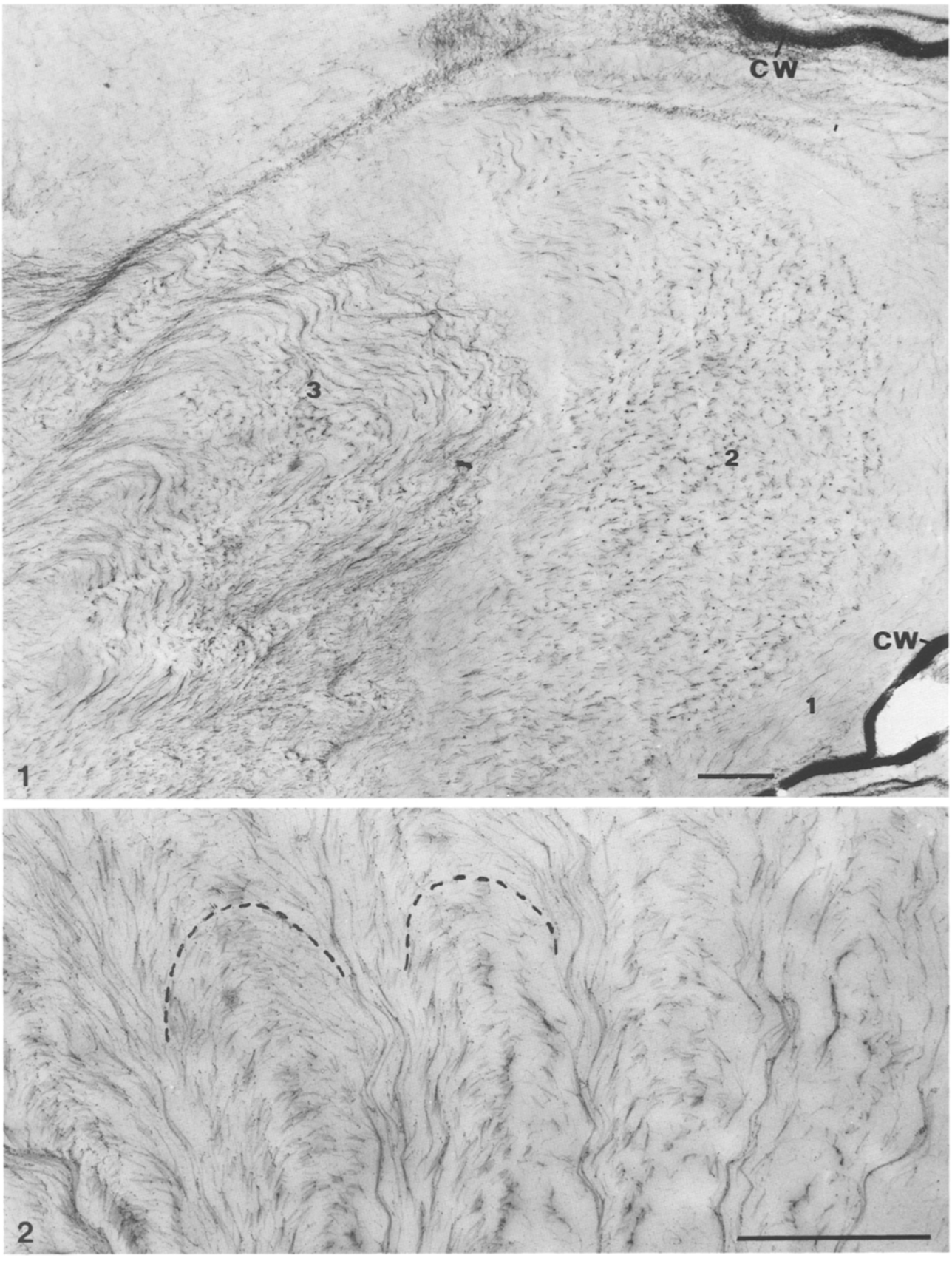
Extraction with trifluoroacetic acid (TFA)

To cellulosic suspension of mucilage (10 mg of lyophilised mucilage in 2.5 ml of distilled water). TFA was added to obtain final concentrations of 1 N, 2 N, 4.5 N, and 8 N. Treatment was made at 40 °C or 60 °C, during 3 h or 6 h. Then, TFA was evaporated from suspension at room temperature during 36 h. The suspension was washed 2-fold with distilled water. It was either directly spread on grids and negatively stained, or incubated with cationic gold and negatively stained as described above.

In order to analyze the influence of pH on flocculation, viscous suspensions (10 mg/2.5 ml, pH 7) were added to 2.5 ml of 1 M Na acetate-HCl buffer or 0.2 M phosphate buffer ranging from pH 1 to pH 8. After stirring, the flocculation state was judged visually and the final pH was measured. The suspensions were then dialized overnight against distilled water in order to check the reversibility of the process.

Estimation of uronic acid composition

Uronic acid was estimated by the meta-hydroxydiphenyl method (Blumenkrantz and Asboe-Hansen 1973) on total mucilage suspension or after TFA treatment. TFA-treated suspension was centrifuged, the supernatant was kept and the pellet was washed and centrifuged, the second supernatant was added to the first and constituted the extracted fraction on which uronic acid was estimated. D-glucuronic acid was used as a standard.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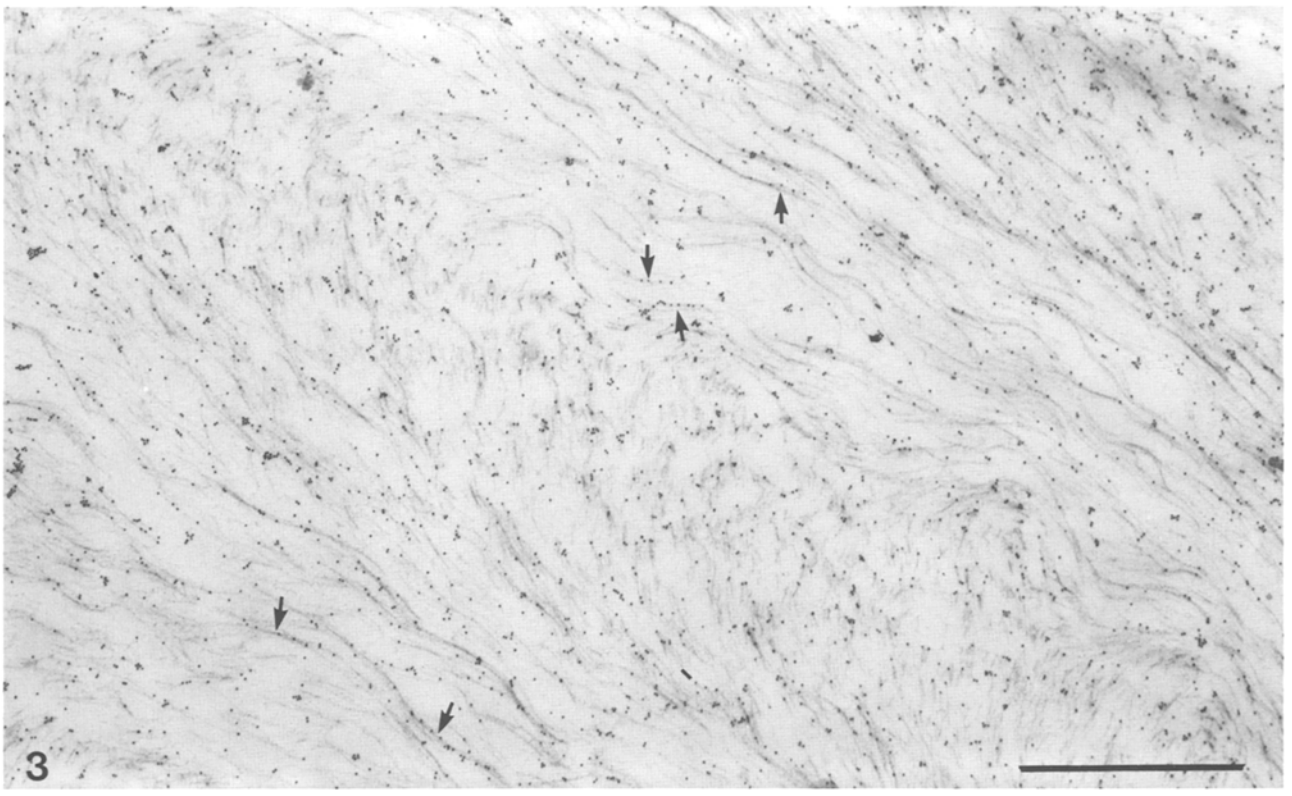


Fig. 3. In situ cytochemical labelling of acidic groups. Quince mucilage. Cationic colloidal gold, uranyl acetate positive staining. Gold particles are regularly aligned along the cellulose microfibrils (arrows), revealing the occurrence of an acidic coat onto the cellulose. Some particles are seen between the microfibrils. Bars: 1 μm

Results

Figures 1–3 remind of some aspects of the organization of the mucilage epidermal cells, when it remains enclosed in an enlarged periplasm, and before it exudes from the cell. At a low magnification (Fig. 1), the whole mass appears typically polydomain. From the periphery to the center of the cell, different aspects are encountered: close to the cell wall, blocks where microfibrils are parallel (1), more internally, blocks without apparent order (2), or stratified and wavy blocks (3). At this level it is difficult to check whether the order is helicoidal. The transition from one block to the other is abrupt. In this enlarged periplasm, ordered blocks are never in contact with the cell wall and the plasma membrane.

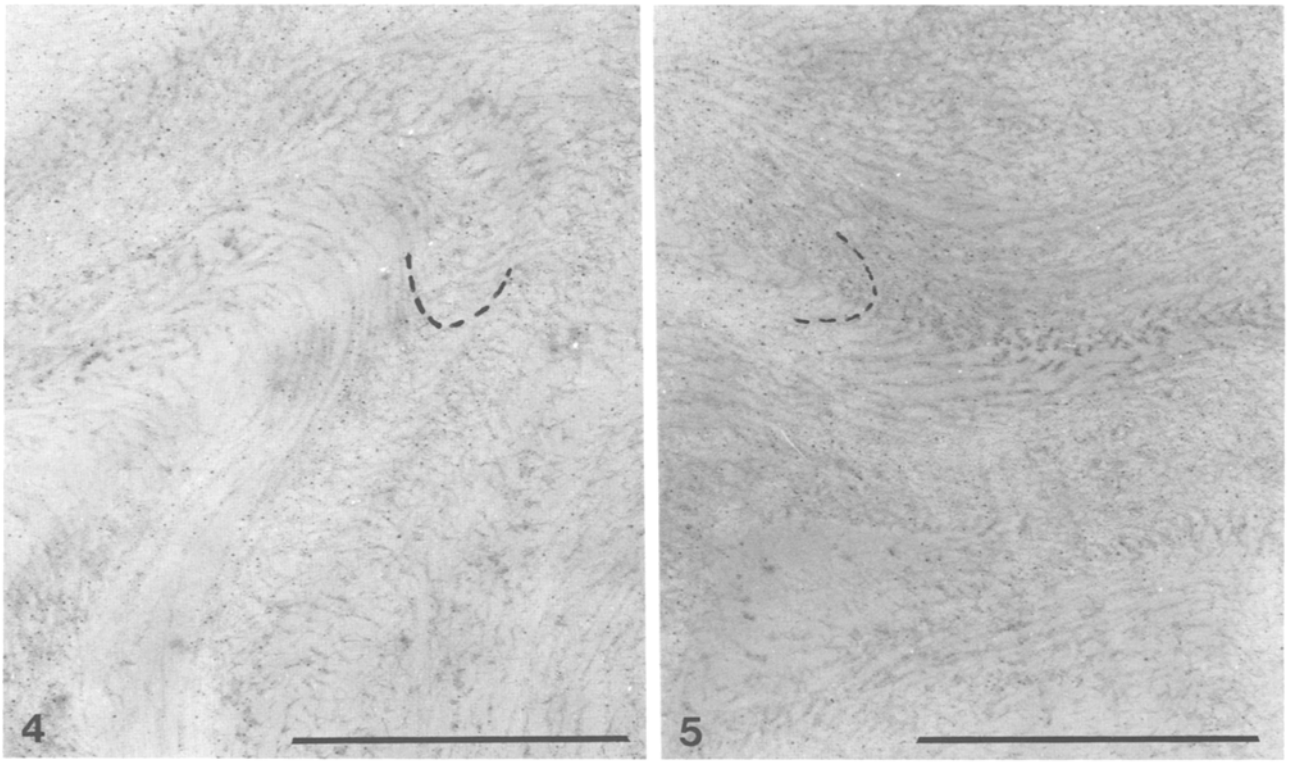
When observed at higher magnification (Fig. 2) the ordered region shows a typical helicoidal organization with successive arcs. The arcs are regular, with a constant amplitude. The fibrillar elements have a sinuous aspect, which is particularly visible when their direction coincides with that of the sectioning plane.

The labelling with cationized colloidal gold, a way to detect the available anionic sites of glucuronoxylan, reveals a regular distribution of the gold particles on the whole mucilage. The majority of particles is aligned

Figs. 1 and **2.** Soft model: quince mucilage. In situ architecture within epidermal cells. PATAg test. Bars: 1 μm

Fig. 1. General view showing the occurrence of several domains within the huge mucilage deposit filling the enlarged periplasm. Close to the cell wall (*CW*) of the epidermal cell, a domain where microfibrils are parallel (*1*). Right, domain without any apparent order (*2*), left, ordered domain with stratified and wavy blocks (*3*)

Fig. 2. Detail of a helicoidal domain. The arcs are clearly seen (dotted line), their general shape is wavy. This is particularly visible when the orientation of the microfibrils coincides with plane of the section



Figs. 4 and **5.** Spontaneous order of in vitro reassembled mucilage suspension. Nanoplast resin, PATAg test. Ordered and stratified domains showing layers in which the microfibrillar subunits vary in orientation (transverse, oblique, longitudinal). Helicoidal-like arcs are seen locally (underlined). In Fig. 4, the whole stratification is deformed in a finger-shaped movement. Bars: 1 μm

along the microfibrils (Fig. 3). Particles are also seen, but in a smaller proportion, in interfibrillar regions.

Reassembly from totally disperse suspensions

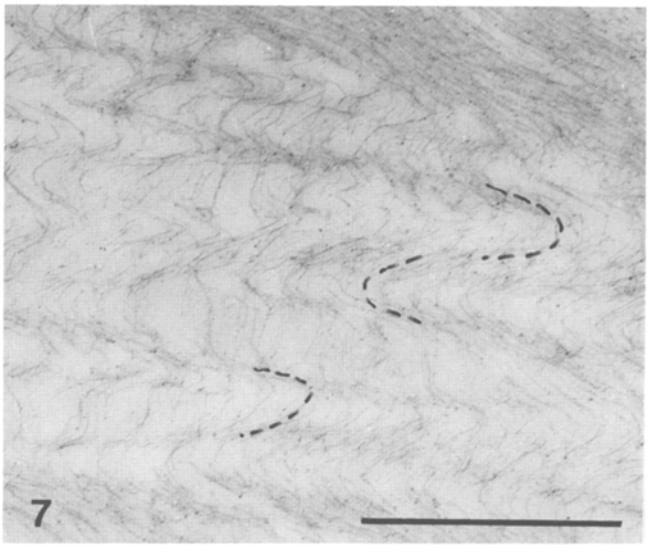
After exuding from the seed epidermis, the hydrated mucilage appears as a homogeneous suspension. It is isotropic when observed with the polarizing microscope. The fibrillar elements are totally dispersed. This is particularly visible with negative staining (Fig. 9) or Pt-shadowing (Fig. 10).

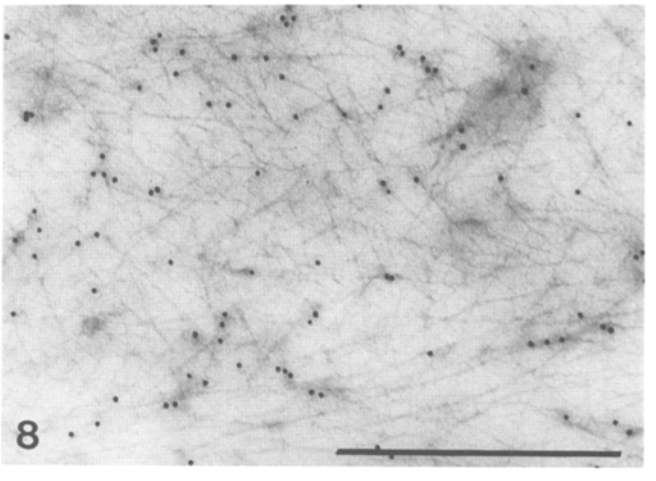
The experiments of reassociation consisted in a progressive elimination of water from the suspension in order to allow the aggregation of the fibrillar elements within a highly concentrated system. This was possible by an ultracentrifugation followed by a gently dehydration at low temperature. It led to the formation of a concentrated viscous drop which was anisotropic in the polarizing microscope. Figures 4 and 5 show the ultrastructural aspect of the reassociated suspensions when the drop was directly embedded in a water-soluble resin (nanoplast), thus avoiding any dehydration step. Images are similar to the native state. The whole system is polydomain, and when an order appears it is seen as series of layers in which the microfibrils are parallel (nematic-like) and oriented in all directions (cholesteric-like) (Figs. 4 and 5). Locally, one can observe arcs. Figure 4 shows a fingershape region with undulating layers, revealing the fluidity of the whole suspension. When the suspension was embedded in more conventional resins, such as LR White that uses steps of alcohol dehydration, the stratified aspect was more clear (Figs. 6 and 7). In Fig. 6 one can see the same type of fingershape undulation as described in Fig. 4. Generally speaking, the fibrillar elements had more contrast and were seen within a clear background. Arcs and sigmoids were more clearly seen (Fig. 7).

Labelling of the reassembled suspension with cationized gold revealed the occurrence of anionic sites along the fibrillar elements (Fig. 8), as in the in situ observations.

Progressive extraction of glucuronoxylan

In order to check the possible intervention of GX in the ordered reassembly of the microfibrils, experiments of progressive extraction of GX were undertaken. They were associated to a control of the fractions both by





Figs. 6–8. Spontaneous order in in vitro reassembled mucilage suspension. LR White resin. Bars: Figs. 6 and 7, 1 µm; Fig. 8, 0.5 µm

Figs. 6 and **7.** PATAg test. The general aspect is similar to Figs. 4 and 5 (stratification, alignments, finger-shaped movements), but the interfibrillar space is increased. In Fig. 7, arcs and sigmoids are clearly visible (dotted lines)

Fig. 8. Cytochemical labelling with cationic colloidal gold. Detail showing the gold particles along the cellulose microfibrils (arrows)

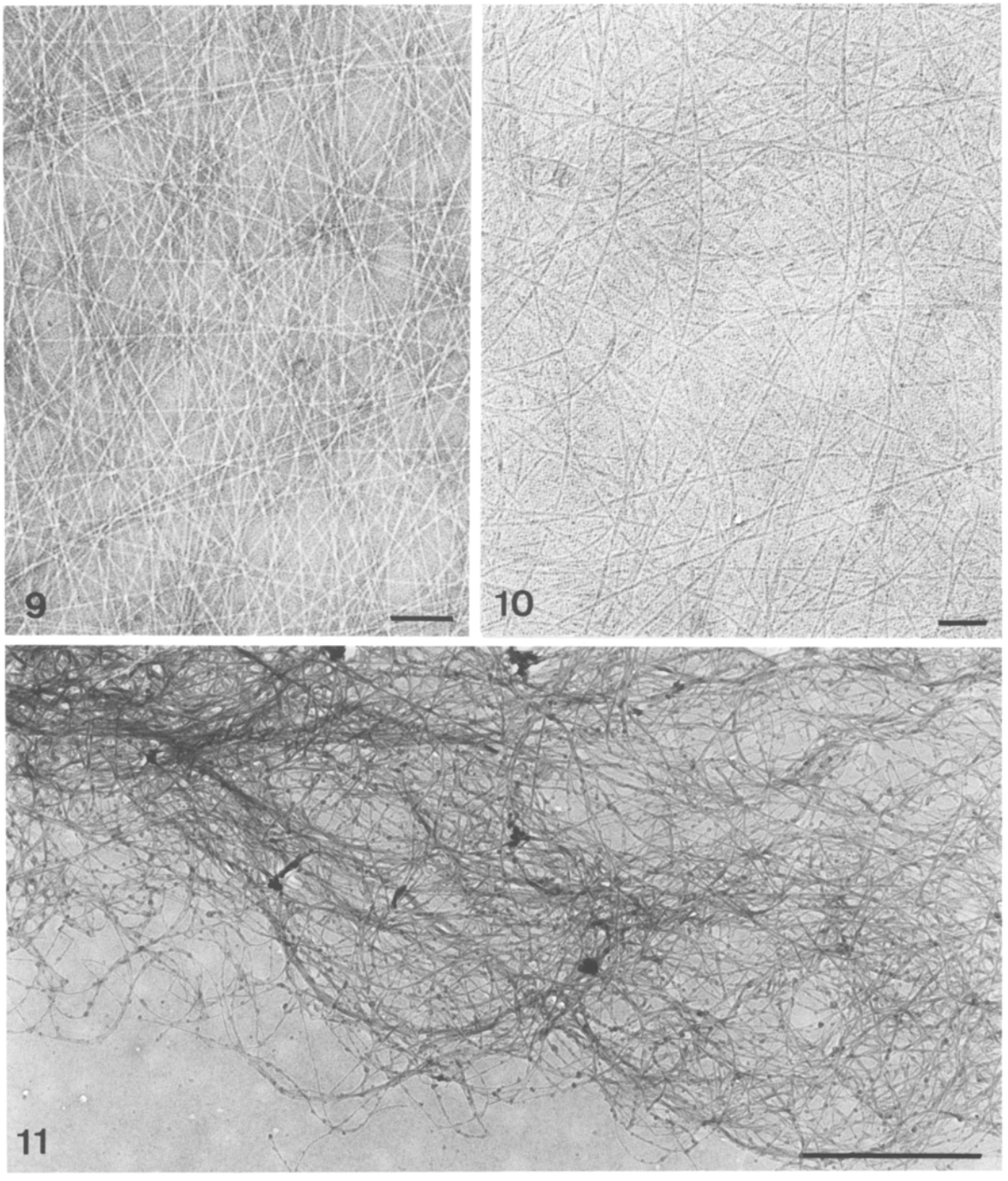
analysis of uronic acid and observation at the electron microscope level.

Table 1. Estimation of uronic acid contents in native suspension and in TFA-extracted fractions

	Native suspension	TFA extracted amount			
		60 °C		40 °C	
		4.5 N, 6 h	2 N, 6 h	N, 3 h	N, 1 h
Glucuronic acid content	26	24.8	17.6	7.25	4.4

Uronic acid content is given in percentage of the whole suspension

In the native suspension, the determination of uronic acid content indicates that the amount of uronic acids in the complete suspension is about 26% (Table 1). The amount of uronic acids extracted by different concentrations of trifluoroacetic acid (TFA) is given in Table 1. It shows in particular that 4.5 N TFA at 60 °C extracts the quasi-totality of uronic acids (24.8% on 26%), whereas 1 N TFA at 40 °C extracts less than 1/5 (4.4% on 26%). The corresponding state of the suspension could be judged visually in the reaction tubes. In all tubes, after addition of TFA, an abrupt change was visible by the formation of two phases: a "floc", i.e., one flocculent mass of microfibrils separated from the solvent and forming a translucent clot. The tubes were put under stirring for variable duration (1 h, 2 h,



Figs. 9–11. Dispersed native suspension and flocculated TFA-treated suspension. Bars: Figs. 9 and 10, 0.1 μm; Fig. 11, 1 μm

Figs. 9 and **10.** Native suspension. Figure 9, negative staining; Fig. 10, rotary Pt-shadowing. The microfibrils are long, linear and thin, regularly dispersed and never aggregated in bundles. Bars: 0.1 μm

Fig. 11. Treatment with concentrated TFA (8 N, 60 °C, 6 h). Negative staining. Flocculation and aggregation of the microfibrils in an entangled network. Bar: 1 μm

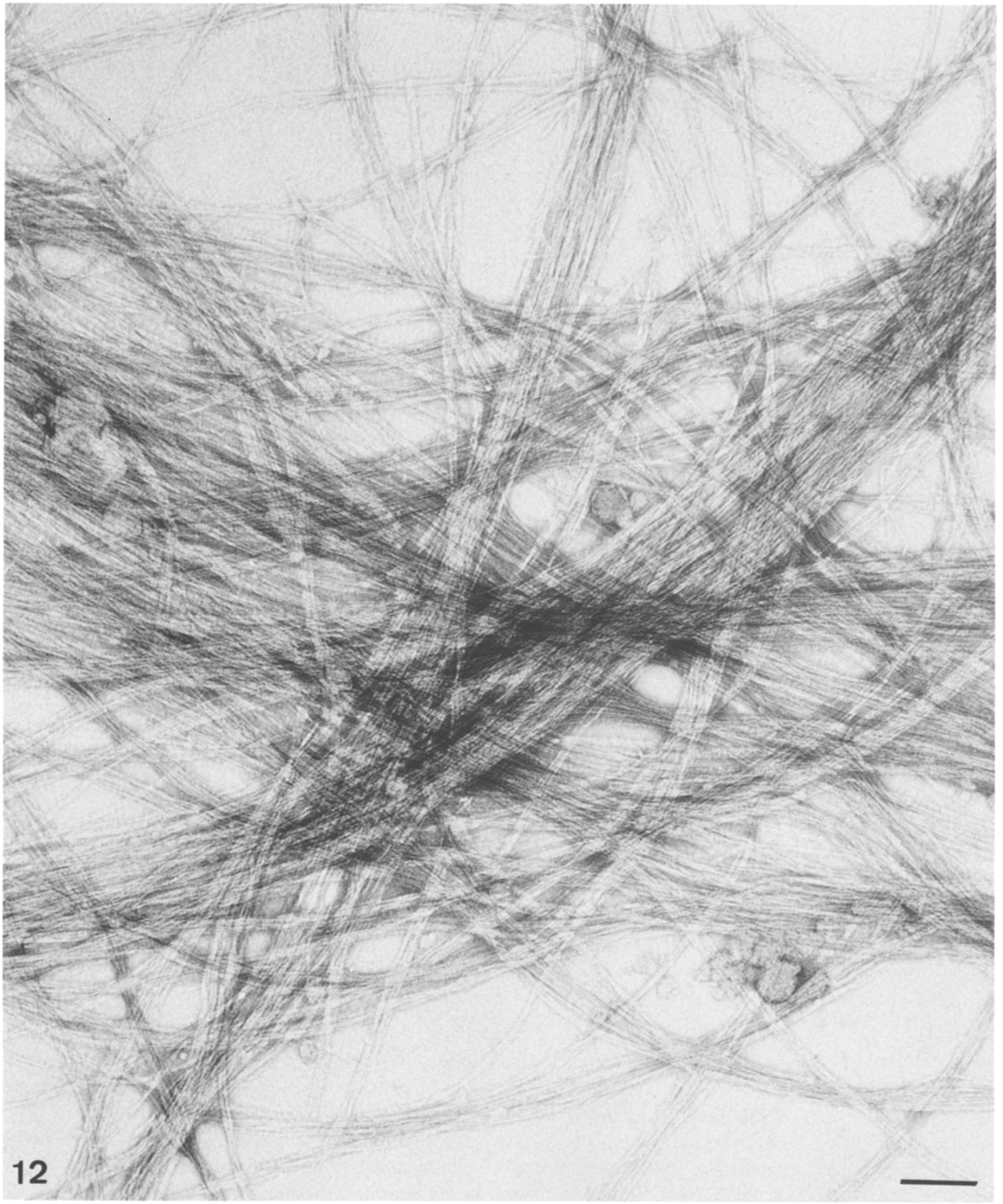
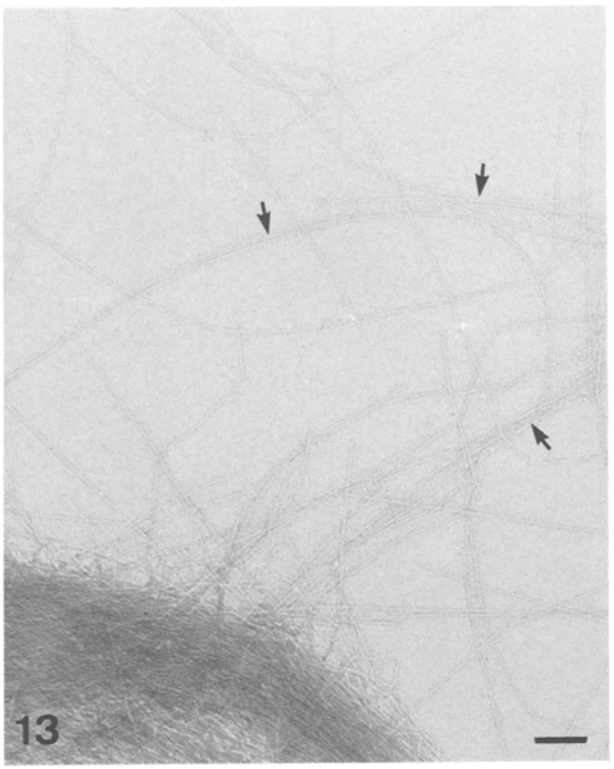
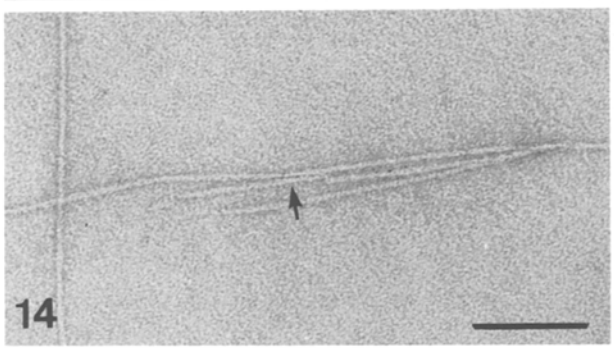


Fig. 12. TFA-treated mucilage suspension (4.5 N, 60 °C, 6 h). Negative staining. The microfibrils are aggregated along their length, forming long bundles of 4–5 subunits minimum. No single microfibrils are seen. Bars: 0.1 μm





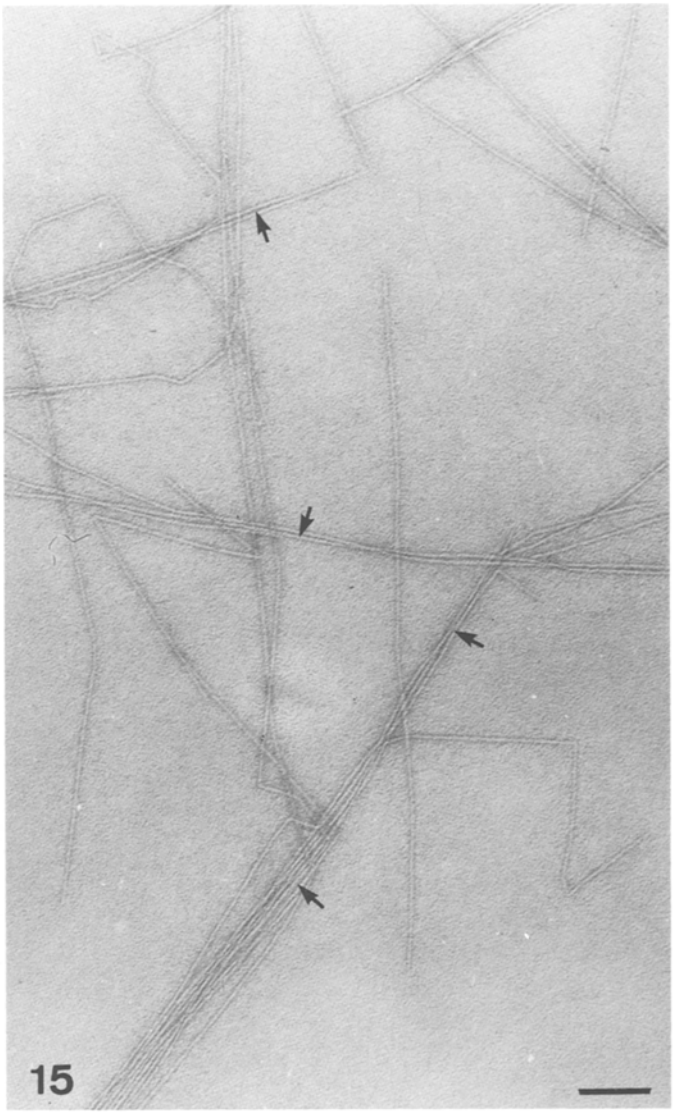


Fig. 13–15. Mucilage suspension treated by mild TFA (2 N, 40 °C, 6 h). Negative staining. Bars: 0.1 μm

Fig. 13. General aspect showing at the same time a dense flocculated zone from which emerge singular or poorly aggregated microfibrils

Figs. 14 and **15.** Details showing singular and linear microfibrils, and all steps of local packing and further association in irregular bundles. In the latter, the microfibrils do not appear tightly packed. Note that ends and kinks of microfibrils are visible

and 6 h) at variable temperatures (20 °C, 40 °C, and 60 °C). They revealed that the more concentrated the TFA, the more intense was the flocculation, with aspects changing from small floccules with 1 N TFA (20 °C, 1 h) to large and indissociable microfibrillar bunches with 4.5 N TFA (60 °C, 6 h). In all cases, ordered helicoidal-like reassemblies could never be obtained from these TFA-treated fractions.

In order to control the possible pH effect of TFA (the solution of TFA is pH 1), experiments were performed in parallel using a range of pH buffer. Below pH 3, a floc appeared with an aspect similar to what was obtained with TFA. The floc was stable but reversibly lost after dialysis against distilled water.

Figure 11 shows the aspect of the flocs obtained with concentrated TFA. It can be compared to the native suspension (Figs. 9 and 10). In the latter, the microfibrils are long, thin (ca. 2 nm in diameter), straight (specially with negative staining), without visible ends, and never aggregated in bundles. Conversely, in the floc, cellulose microfibrils are randomly intermingled forming a mass from which fibrillar bundles of various sizes

escape (Fig. 11). At high magnification one can see clearly that microfibrils are associated along a great length (Fig. 12). The number of subunits per bundle is highly variable, but at least 4–5. No isolated microfibril was ever seen.

Figures 13–15 show the intermediate steps of aggregation with less concentrated TFA. The large entangled mass is seen but also all the steps ranging from single subunits to local association in irregular bundles. Whatever the situation, the microfibrils are not packed along their whole length. From place to place, microfibrils are broken and present kinks. At pH 2 and without extraction, flocculation also occurred, microfibrils were packed forming a compact network, but without close association of microfibrils together (data not shown).

Discussion

Polydomain organization of in vitro reassemblies

The present results confirm that it is possible to reobtain a cell wall-like organization provided that suitable experimental conditions are found: elimination of water, concentration of the suspension, and facilitation of the closeness of subunits so that they can interact. One aspect of these experiments, i.e., the possibility to compare the results of reassociation to what can be observed in vivo, leads to a revaluation of the mechanism of cell wall assembly. In particular, experiments show that the assembly depends not only on the properties of the polymers themselves but also on the conditions of the environment.

In the great majority of plant cells, the periplasm is a thin space maintained under turgor pressure and in which the polymers that are continuously synthesized by synthetases are very concentrated beyond the plasmalemma. Physico-chemical conditions are complex and difficult to precisely analyze. The previously assembled cell wall is close to the plasmalemma and constitutes a barrier or a constraining layer for the polymers that are in the course of assembly (Neville 1988 b). The result is typically a monodomain construction.

The epidermal cells of quince provide an exceptional case where an enlarged periplasm exists in physiological conditions (and not in plasmolysis conditions). In this periplasm, the cellulosic mucilage accumulates with time, far from cellular constraints (cell wall and plasmalemma). At first, the mucilage concentration is low, and it appears dispersed, without apparent order. When the mucilage is more concentrated, an order appears as nodules well away from the cell wall and the plasmalemma. It is polydomain with some domains looking like nematic liquid crystals and other domains looking like cholesteric liquid crystals. When the mucilage concentration increases, it becomes more organized and seems to tend to a monodomain.

In vitro experiments constitute an extreme case where there is no constraining surface. Experimentally induced constraints are the centrifuge forces and controlled dehydration which restricts the total volume of the suspension. On the whole, the suspension is dispersed. An order appears locally when the concentration conditions are more favourable and is seen as separated nodules within a more or less dispersed suspension. The system is polydomain. When an order is visible, it is cholesteric-like and presents transition states with many imperfections. Other systems of in vitro liquid crystal reassemblies have been tested from biological molecules: collagen (Giraud-Guille 1992), DNA (Leforestier and Livolant 1991), microfibrillar cellulose (Revol et al. 1992, 1994). The resulting organization often is typically cholesteric. In all cases, the experiments were carried out with fragmented molecules (chemically or following ultrasonication), 20–200 nm in length. Here the fibrillar elements (cellulose coated with GX) are very long (no visible ends in native suspension) and dispersed, therefore difficult to reassemble in a perfect order. They have to establish both an alignment and a twist in the suspension. The process is accelerated by the centrifugation that likely helps the microfibrils to prealign. In the periplasm, the microfibrils are secreted along the plasmalemma with likely a first two-dimensional alignment parallel to this planar surface. This aspect has been clearly emphasized by Giraud-Guille (1992) for in vitro reassembly of collagen molecules; the author showed that when a prealignment is acquired the twisted geometry is much more easy to obtain with long molecules.

Ultrastructural variability due to the preparation procedure

Quince mucilage is a highly hydrophilic material, and possible artefacts can be produced during dehydration steps. To check this technical aspect, two resins were used: a conventional resin (LR White) and a water-soluble melamin resin (nanoplast). The results show that the obtained images are slightly different even though the general order (domains with cholesteric organization) is maintained. In LR White, the stratified and helicoidal-like aspect is obvious, the whole fibrillar elements appear sharp, and the pitch between the layers

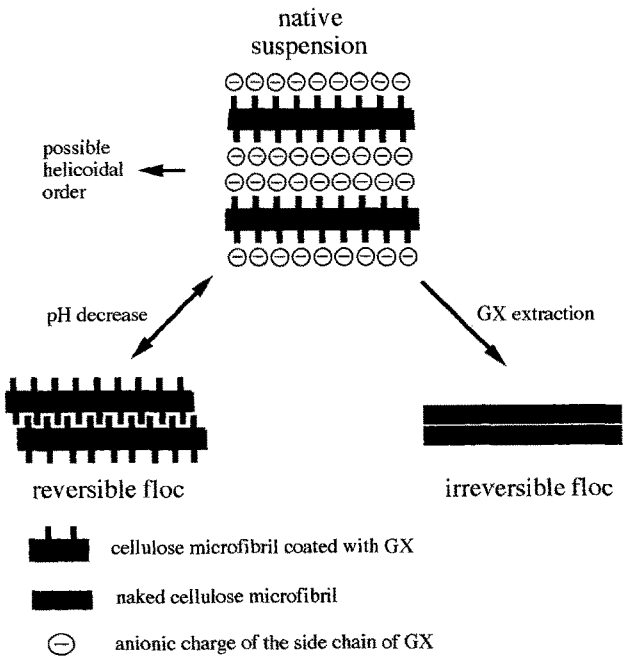


Fig. 16. Schematic representation of the antifloc role of GX. In native conditions, a helicoidal order arises from GX-coated cellulose microfibrils that are maintained spaced by the electrostatic repulsion of the side-chains of the GX. A pH decrease (cancellation of the electrostatic repulsion) provokes a flocculation that is reversible. Conversely, a removal of GX provokes an irreversible flocculation of the suspension

is smaller than after inclusion with nanoplast. Such images are very close to those obtained in situ, with specimens embedded in the same resin (LR White).

It is likely that the steps of dehydration through the alcohol series provokes a general compaction of the polysaccharides, mainly the chains of GX. In native conditions, the microfibrils are likely enveloped by a tightly bound GX layer and surrounded by a less tightly bound GX layer forming a cloud spread out because of the electrostatic repulsions. With the alcohol treatment, even mild and progressive, all chains artefactually flatten onto the surface of the microfibrils leading to a better exposure of the texture. The removal of water is achieved without a serious disruption of the initial order since the order remains the same in both cases. A similar phenomenon has clearly been described in solid films obtained from helicoidal suspensions of cellulose crystallites prepared from wood pulp (Revol et al. 1992).

GX as antifloc and/or twisting agents?

In previous papers, we had postulated that GX could play a key role in helicoidal assembly. The question remains to know whether this role is mainly an antifloc role due to the anionic charges or whether the conformation of GX provides also the capacity for cholesteric rotation of the microfibrils.

Several pieces of data show that the antifloc role of GX has to be taken into account: (1) a decrease of pH of the cellulose suspension (pH lower than 3) provokes a flocculation that is reversible (Fig. 16); (2) a controlled chemical extraction of the GX from the suspension also provokes a flocculation, but that is irreversible whatever the pH (Fig. 16). The more extracted the GX, the more important is the floc; (3) the observation of suspensions at high resolution allows to relate the progressive extraction of GX and the occurrence of microfibril/microfibril junction zones. The latter are all the more long and frequent as the extraction is more pronounced, and to some extent images lead to the formation of indissociable bundles, whereas a simple change in pH provokes a compaction of microfibrils but neither junction zones nor bundles. This clearly demonstrates that GX are antifloc agents that allow the microfibrils both to be spaced and to remain spaced, conditions that create a fluidity necessary for the establishment of a cholesteric order.

The ultimate question is to know what is the cause of the cholesteric order. It cannot be excluded that it could come from cellulose itself due to its chiral character. That could be postulated in the experiments of Revol et al. (1992, 1994) from suspensions of cellulose of wood paper, in which small crystallites likely ionized during the preparation treatment leading to typical cholesteric liquid crystal assemblies. In the present system, though charged by the GX coat, the microfibrils are likely too long to spontaneously re-order owing only to their chirality. Therefore the function of GX as helper twisting agent must not be ruled out in addition to its evident antifloc role. As it has been claimed in the literature, in many systems, polysaccharidic or not, the shape of the molecules is important. According to Neville (1993) the molecular shape is important to generate helicoids by self-assembly, particularly if it is helical and pipe-cleaner type. Here, in the molecule of GX, one finds the credentials required to form a cholesteric: "stiff backbone, bulky flexible side chains allowing the backbone to move as if it were in a liquid crystal, asymmetric carbon in each carbon residue" (Gray 1983; Neville 1988 a, 1993).

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