In Vitro Versus in Vivo Cellulose Microfibrils from Plant Primary Wall Synthases: Structural Differences*

Received for publication, April 12, 2002, and in revised form, July 25, 2002
Published, JBC Papers in Press, July 26, 2002, DOI 10.1074/jbc.M203530200

Joséphine Lai-Kee-Him‡, Henri Chanzy‡, Martin Müller§§, Jean-Luc Pautaux‡, Tomoya Imai‡§, and Vincent Bulone‡**

From the ‡Centre de Recherches sur les Macromolécules Végétales (CERMAV-UPR CNRS 5301), Joseph Fourier University of Grenoble, B.P. 53, 38041 Grenoble cedex 9, France and the §European Synchrotron Radiation Facility, B.P. 220, 38043 Grenoble, France

Detergent extracts of microsomal fractions from suspension cultured cells of Rubus fruticosus (blackberry) were tested for their ability to synthesize in vitro sizable quantities of cellulose from UDP-glucose. Both Brij 58 and taurocholate were effective and yielded a substantial percentage of cellulose microfibrils together with (1→3)-β-D-glucan (callose). The taurocholate extracts, which did not require the addition of Mg2+, were the most efficient, yielding roughly 20% of cellulose. This cellulose was characterized after callose removal by methylation analysis, electron microscopy, and electron and x-ray synchrotron diffractions; its resistance toward the acid Updegraff reagent was also evaluated. The cellulose microfibrils synthesized in vitro had the same diameter as the endogenous microfibrils isolated from primary cell walls. Both polymers diffracted as cellulose IV, a disorganized form of cellulose I. Besides these similarities, the in vitro microfibrils had a higher perfection and crystallinity as well as a better resistance toward the Updegraff reagent. These differences can be attributed to the mode of synthesis of the in vitro microfibrils that are able to grow independently in a neighbor-free environment, as opposed to the cellulose in the parent cell walls where new microfibrils have to interweave with the already laid polymers, with the result of a number of structural defects.

The organized deposition of cellulose in plant cell walls is one of the most important biochemical phenomena during cell growth and differentiation. The process of cellulose biogenesis requires a number of steps that occur in a coordinated fashion, from the polymerization of long flawless (1→4)-β-D-glucan chains to their precise assembling and extrusion as slender monocrystalline microfibrils into the cell wall matrix (1–5). Despite the ubiquity of cellulose in plant cell walls and the relative simplicity of the molecular structure of this polymer, its biosynthesis is far from being fully understood, although substantial progress has recently been made in the genetics (6–10), the biochemistry (11–16), and the regulation (17–19) of cellulose synthases. In particular, one has not yet reached the point where sizable amounts of plant cellulose microfibrils can be synthesized in vitro, in contrast with other biologically important polysaccharides such as plant and fungal (1→3)-β-D-glucans (13–15, 20–24) or fungal α-chitin (25, 26), for which significant quantities of in vitro microfibrillar polymers can be readily prepared.

The intrinsic nature of native cellulose (or cellulose I) microfibrils where all of the chains are organized in parallel fashion appears to be due to the organization of cellulose synthases in the plasma membrane. It seems now accepted that these membrane proteins are organized as rosette-like structures (7, 27–31), designated as terminal complexes, that act as spinnerets for the extrusion of microfibrils. Apparently, it is only when the integrity of this machinery is kept intact, as in the plant plasma membrane, that all of its proteinaceous subcomponents are able to polymerize glucosyl units in a coordinated fashion, leading to the spinning of cellulose I microfibrils (7). Other systems where the enzymes that synthesize cellulose are not arranged in organized terminal complexes tend to produce cellulose II whose structure and morphology differ from those of native microfibrils (32–34).

Given the instability of the cellulose synthase complexes, their extraction from plasma membranes and their use to produce in vitro cellulose I have proven quite challenging. In fact, when incubated with UDP-glucose, plant membrane extracts usually yield quantities of (1→3)-β-D-glucan but no cellulose (22–24). It is only in very few instances that a careful fractionation of membrane extracts has led to the preparation of minute quantities of cellulose I in the middle of large amounts of (1→3)-β-D-glucan (13–15). In these experiments, cellulose could be detected by fluorochrome and radiochemical labeling. In addition, ultrastructural observations by transmission electron microscopy (TEM) have revealed the occurrence of very long, slender microfibrils, dispersed in the preparation. These microfibrils were identified as cellulose by immunogold labeling and/or electron diffraction analysis (13–15).

Despite the hopes brought by the first in vitro syntheses of cellulose I from plant membrane extracts (13–15), the full description of the polymer requires the biosynthesis and purification of a sizable amount of product. Indeed, milligrams of purified sample are necessary to obtain reliable structural information on a given polymer. With this in mind, the aim of the present work was to produce relatively large quantities of in

---

* This work was supported by a grant-in-aid (to V. B.) from Hercules Inc. (Wilmington, DE). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

** Present address: Institut für Experimentelle und Angewandte Physik der Universität Kiel, Leibnizstr. 19, D-24098 Kiel, Germany.

† Recipient of fellowships from the French Ministry of Research and from CNRS.

§ To whom correspondence should be addressed: Laboratoire de Physico-Chimie Biologique, UMR CNRS 5013, Bâtiment Chevreul 4ème étage, Université Claude Bernard Lyon I, 43 Blvd. du 11 Novembre 1918, 69622 Villeurbanne cedex, France. Tel.: 33-4-72-43-15-42; Fax: 33-4-72-43-15-43; E-mail: Vincent.Bulone@univ-lyon1.fr.

1 The abbreviations used are: TEM, transmission electron microscopy; Mops, 4-morpholinoethanesulfonic acid; GC, gas chromatography; MS, mass spectrometry.
in vitro cellulose from detergent extracts of plant microsomes. We isolated cellulose synthases from *Rubus fruticosus* (blackberry) suspension-cultured cells and selected biosynthesis conditions that led to the preparation of in vitro cellulose in quantity suitable for chemical, biochemical, and physical characterization. These characterizations revealed the differences existing between the in vitro synthesized polymer and the endogenous cell wall cellulose.

**MATERIALS AND METHODS**

*Chemicals*—Taurocholate, Brij 58, Mops, UDP-glucose, and cellulose were purchased from Sigma. UDP-β-[U-14C]glucose (300 mCi/mmol) and the liquid scintillation mixture were bought from Isotopim (Feyrius, France) and Amersham Biosciences, respectively. All other chemicals, including the medium used for suspension cultures, were from Sigma.

*Suspension Cultures of *R. fruticosus* Cells*—Cells from *R. fruticosus* (35) were maintained as suspension cultures in Heller medium (36). They were grown at 23 °C under agitation on a rotary shaker (120 oscillations/min) and successive 12-h night and day periods. Cells harvested in stationary phase after 18–24 days of culture were washed thoroughly with distilled water and used for isolation of the microsomal fraction.

*Preparation of Microsomal Membranes and Detergent Extraction of Glucan Synthases*—Cells were disrupted at 80 megapascals using a French press (SLM-Amino Instruments, Thermo Spectronic, Rochester, NY), and the microsomal fraction was isolated by differential centrifugation as previously described (24). Taurocholate or Brij 58 were used at their critical micellar concentrations, 0.3 and 0.05%, respectively, to extract the membrane-bound proteins (−4 nmol, final concentration). After a 30-min incubation at 4 °C in the presence of either of the two detergents and under continuous stirring, the microsomal membranes were centrifuged at 150,000 g for 1 h. The supernatant was used as the detergent-extracted enzyme preparation. (1→3)-β-Glucan and cellulose synthase activities were stable for several weeks when the fractions were kept at 4 °C.

**Determination of the Yield of in Vitro Synthesis of Cellulose and (1→3)-β-Glucan**—To determine the yield of cellulose synthesis, radioactive products were synthesized in vitro in the presence of UDP-β-[U-14C]glucose and incubated with enzymes that specifically hydrolyze cellulose or (1→3)-β-glucan. A typical in vitro synthesis reaction consisted of a mixture of 6 ml of either of the two detergent extracts, 12 ml of 37°C Mops-NaOH buffer, pH 6.8, containing 40 mM cellobiose, 1.2 ml of 20 mM nonradioactive UDP-glucose (i.e. 24 μmol of UDP-glucose) and 2.9 μCi of UDP-β-[U-14C]glucose. The volume of the reaction mixture was completed to 24 ml with distilled water. The molar ratio between the total UDP-glucose and the 14C-labeled UDP-glucose was 36932:1. The experiments described above for the determination of the yields of in vitro cellulose and (1→3)-β-glucan were repeated six times in order to demonstrate the reproducibility of the synthesis reactions. The average and S.D. values obtained from these experiments are presented in Table I.

**Large Scale Synthesis of Cellulose**—As indicated in Table I, the highest yield of in vitro cellulose was obtained using taurocholate extracts. When 6 ml of these detergent extracts were used for in vitro synthesis, the equivalent of up to 25 μg of glucose was incorporated into cellulose. For the large scale production of cellulose, a typical synthesis was performed from 60 g of fresh cells. This allowed the preparation of 212 mg of cellulose from taurocholate extract, which yielded, according to the data presented in Table I, nearly 100 μg of cellulose. Such large scale syntheses were repeated 10 times in order to accumulate enough cellulose (~1 mg) for structural characterization.

**Purification of the in Vitro Cellulose**—Although the conditions described above were optimal for the synthesis of cellulose, (1→3)-β-glucan was the dominant polysaccharide synthesized in the reaction mixture. In order to characterize the cellulose produced in vitro, it was therefore necessary to eliminate the (1→3)-β-glucan. The in vitro products were first incubated for 2 h at room temperature in chloroform/methanol (2/1, v/v) and then washed five times by centrifugation with distilled water. The (1→3)-β-glucan was selectively solubilized by incubating the preparation in a 3% NaOH solution at 4 °C for 2 h. The resulting product was washed with distilled water to reach neutrality and incubated in the Updegraff reagent (39) at 100 °C for 30 min. After extensive dialysis against distilled water, the resulting cellulose was kept at 4 °C as a suspension in 0.01% sodium azide before TEM observation. For the other analyses (x-ray diffraction and methylation analysis), the cellulose was freeze-dried.

**Preparation and Purification of the Plant and Bacterial Celluloses Used as Controls**—Twenty-one-day-old *R. fruticosus* cells were washed thoroughly with distilled water and disrupted in a French pressure cell at 80 megapascals, as previously described (24). The homogenate was centrifuged at 5000 × g for 10 min. The pellet containing unbroken cells and primary walls was extracted by a 5% KOH solution during 48 h at room temperature under continuous mild stirring. The mixture was centrifuged at 5000 × g for 10 min, and the pellet was dispersed in 50 mM acetate buffer, pH 5, containing 1.7% sodium chloride. This suspension was heated during 8 h at 80 °C, and the treatment was repeated twice. The insoluble cellulose composing the cell wall was recovered after centrifugation at 5000 × g for 10 min, washed with distilled water, and extracted with 3% NaOH for 12 h at 4 °C. The insoluble material was centrifuged (5000 × g, 10 min), washed with distilled water until a neutral suspension was obtained, and centrifuged again before treatment with the Updegraff reagent (39) for 30 min at 100 °C. The mixture was extensively dialyzed against distilled water, and the cellulose recovered by centrifugation (5000 × g, 10 min) was used as a control for the following analyses.

Bacterial cellulose was extracted from *Acetobacter xylinum* cultures prepared in a peptone-rich medium containing yeast extract and glucose. The cellulose resulting from 1 month of culture was thoroughly washed under tap water for 1 week and then successively washed with 1% NaOH, 1% HCl, and distilled water. It was finally treated with the Updegraff reagent (39) for 30 min at 100 °C and extensively dialyzed against distilled water. The cellulose recovered by centrifugation (5000 × g, 10 min) was stored at 4 °C in distilled water containing 0.01% sodium azide.

**Cellulose Methylation Analysis**—The purified cellulose samples treated with the Updegraff reagent were permethylated and analyzed for linkage type by the alditol acetate method of Harris et al. (41). The methylated monosaccharides released by trifluoroacetic acid hydrolysis were reduced with NaBD4 and acetylated. The methylated alditol acetates were separated by gas chromatography (GC) with a 30 m × 0.32 mm glass column (Supelco), with a temperature program increasing from 165 to 225 °C at a rate of 2.5 °C min⁻¹. The GC peaks were identified by electron impact mass spectrometry at an ionizing potential of 70 eV, using a Nermag R10–10C mass spectrometer connected to a Delsi Di 700 GC fitted with a capillary SP 2380 column.

**Purification and 13C NMR Spectroscopy of (1→3)-β-D-Glucan**—The (1→3)-β-D-glucan present in the biosynthetic mixture was purified and characterized as follows. The sodium salt was lyophilized for 18 h at 37 °C with protease K (6 units/mg of polysaccharides) in 10 mM Tris/HCl, pH 7.5, and recovered by centrifugation (5000 × g, 10 min). Lipids and peptides were successively extracted with distilled water, 66% methanol, and chloroform/methanol (1:2, v/v). These extractions were repeated four times. The in vitro products collected by centrifugation were washed thoroughly with water and treated for
**In Vitro Synthesis of Cellulose by Plant Cell-Free Extracts**

**Table I**

| Table I | Yields of in vitro cellulose and (1→3)-β-D-glucan as a function of the detergent used for extraction of the glucan synthases from R. fruticosus membranes |
|---------|---------------------------------------------------------------------------------------------------------------------------------|
| Detergent extract used for in vitro synthesis | Polysaccharide synthesized in vitro | dpm incorporated | pmol of radioactive glucan incorporated | Total nmol of glucose incorporated | Total μg of glucose incorporated | Percentage of in vitro products |
|---------|---------------------------------------------------------------------------------------------------------------------------------|----------------|---------------------------------|-------------------------------|--------------------------------|--------------------------------|
| Brij 58 extract | Cellulose | 21,445 ± 6971 | 33.4 ± 10.8 | 80.5 ± 26.2 | 13.0 ± 4.2 | 16.2 ± 5.2 |
| | (1→3)-β-D-Glucan | 111,098 ± 29,999 | 173.0 ± 46.6 | 417.2 ± 112.4 | 67.6 ± 18.2 | 83.8 ± 22.5 |
| Total | 132,533 ± 11,962 | 206.4 ± 18.6 | 497.7 ± 44.9 | 80.6 ± 7.3 | 100 ± 9.1 |
| Taurocholate extract | Cellulose | 38,994 ± 3824 | 60.7 ± 5.9 | 146.5 ± 14.4 | 23.7 ± 2.3 | 20.0 ± 1.9 |
| | (1→3)-β-D-Glucan | 156,559 ± 4275 | 243.9 ± 6.7 | 588.0 ± 16.0 | 95.3 ± 2.6 | 80.1 ± 2.2 |
| Total | 195,553 ± 4210 | 304.6 ± 6.6 | 734.4 ± 15.8 | 119.0 ± 2.6 | 100 ± 2.2 |

**Results**

**Biochemical Characterization of the in Vitro Products**—A series of detergents were screened for their ability to extract cellulose synthases in an active form from plasma membranes. Taurocholate and Brij 58 used at their critical micellar concentrations were the most efficient detergents for in vitro synthesis of cellulose. However, the reaction mixture recovered after synthesis with Brij 58 or taurocholate extracts also contained (1→3)-β-D-glucan was deposited on a carbon-coated stainless steel grid and allowed to dry. The (1→3)-β-D-glucan was selectively removed by immersing the grid into a solution of 3% (w/v) NaOH. The grid was then washed with methanol and subjected to the biotinylination reaction before coupling with streptavidin-gold. The grid was washed in distilled water, dried, and shadowed with W/Ta prior to TEM observation.

**Synchrotron X-ray Diffraction Analysis**—The following samples were analyzed: (i) a sample of in vitro cellulose after Updegraff treatment; (ii) a sample of cellulose extracted from R. fruticosus cell walls after disaccharidase and Updegraff treatment; (iii) a sample of bacterial cellulose treated with Updegraff reagent. All samples were washed with distilled water and freeze-dried. Small amounts of these samples were then mounted across the hole of holders that consisted of thin rigid cardboard disks of 1.5-cm diameter, perforated in their center with a 2-mm diameter hole. These sample holders were then positioned on a goniometer head.

**Six replicates were carried out for each condition to obtain S.D. values.**

The optical axis of the micrograph and that of the beam was 70°. This orientation was calibrated with an accuracy of better than 1 μm. Two-dimensional diffraction patterns were recorded on a CCD detector (2048 × 2048 pixels; MAR Research) at a sample to detector distance of 57 mm. The detector had a pixel size of 64.45 × 64.45 μm² and a dynamic range of 16 bit. Corundum powder in a glass capillary was used for calibration.

Each of the three samples was sequentially translated through the beam in steps of 10–30 μm, in order to sample over the whole specimen area. At every position, a two-dimensional diffraction pattern was acquired during 60 s. Background images were collected as well, and the data analysis (e.g., background removal, averaging, azimuthal integration, etc.) was achieved with the ESRF image processing software FIT2D (44). The diffraction diagrams were fitted with a least-squares algorithm. The peak shape was assumed to be Lorentzian (45), and the peak positions were calculated using cellulose II cell parameters (46). The lattice parameter a, the reflection widths, and a linear background were the only variable parameters in the fit. The peak widths (expressed as 2θ angle) were used to calculate the apparent lateral crystal sizes, using the Scherrer equation (47). The relative x-ray crystallinity of each sample was obtained by computing the ratio of the intensity of the 200 reflection and the total scattering intensity at this position in the diagram (i.e., the sum of the peak intensity and the amorphous background). Our values of crystal size and crystallinity obtained with bacterial cellulose are in agreement with the data in the literature (48, 49).
thesis of only 16% cellulose (Table I). In addition, the amount of cellulose synthesized in vitro expressed as µg of glucose incorporated into the polymer was on average 2 times higher with taurocholate extracts than with Brij 58 extracts (Table I). Indeed, the use of 6 ml of taurocholate extract and a final reaction mixture of 24 ml containing the equivalent of 4.3 mg of glucose allowed the synthesis of up to 25 µg of cellulose, compared with 13 µg with Brij 58 extracts (Table I). From this result, we can predict that large scale synthesis reactions performed using 25 ml of taurocholate extract yield about 100 µg of cellulose. Since 10 runs of synthesis in these large scale conditions were carried out, we can estimate that the total amount of cellulose synthesized in vitro is about 1 mg. Similarly, since the reaction mixtures after in vitro synthesis contained on average 80% of (1→3)-β-D-glucan (Table I), it can be estimated that the total amount of (1→3)-β-D-glucan produced in the large scale conditions is 3.8 mg. Interestingly, the presence of Mg$^{2+}$ was required for cellulose synthesis when Brij 58 was used to extract the cellulose synthase, whereas the yield of cellulose was higher in the case of taurocholate extracts when no Mg$^{2+}$ was added.

The structure of the (1→3)-β-D-glucan present in the reaction mixture was confirmed by $^{13}$C NMR spectroscopy. The spectrum obtained after dissolution of the polysaccharide with (CD$_3$)$_2$SO (Fig. 1) contains six sharp peaks at 60.9, 68.4, 72.8, 76.3, 86.2, and 103.0 ppm. It is characteristic of a strictly linear (1→3)-β-D-glucan (50–52). In addition, there is no hint of peaks that can be attributed to the chain ends. Altogether, these data indicate that this polymer is linear and of a relatively high molecular weight.

Chemical Characterization—One part of the purified in vitro cellulose treated with the Updegraff reagent was subjected to methylation analysis, and the resulting permethylated alditol acetates were identified by GC/MS. The gas chromatogram presents only one intense peak that was identified as corresponding to 1,4,5-tri-O-acetyl-2,3,6-tri-O-methyl-D-glucitol by electron impact MS (Fig. 2A). The yield of cellulose was deduced to be 16%, and it is not clear whether some of these are attached to the tips of the microfibrils. The lateral size of the microfibrils appears to be constant. However, giving their width with ac-
observed after incubation with 1 mM UDP-glucose. Branes from R. fruticosus minimum volume (freeze-dried, and the resulting material was resuspended in a water) to prepare this control, 10 ml of a taurocholate extract were used for TEM observation. To test very rarely revealed the presence of some microfibrillar polymers that were found to be sensitive to the Updegraff treatment (not shown), whereas the sample obtained after in vitro synthesis systematically contained microfibrillar cellulose, as illustrated in Figs. 4C and 6. These data confirm that the observed cellulose was not arising from the original microsomes but was indeed newly synthesized by the detergent extract used as a source of enzyme.

A comparison of the ultrastructure of the endogenous and in vitro celluloses is presented in Fig. 4, where all the micrographs were recorded from negatively stained specimens. Fig. 4A corresponds to a purified preparation of endogenous cellulose that had not undergone the Updegraff treatment. As expected, this cellulose consists of a bundle of parallel endless thin microfibrils only about 2–3 nm in diameter. After Updegraff treatment, these microfibrils kept their initial lateral width, but they were drastically cut longitudinally into fragments of no more than 200–300 nm in length (Fig. 4B). In addition, the sample in Fig. 4B is no longer organized in bundles but occurs as small packets of 3–10 elements. A sample of in vitro cellulose recovered after Updegraff treatment is shown in Fig. 4C. As opposed to the corresponding endogenous cellulose (Fig. 4B), the Updegraff-treated in vitro cellulose consists of much longer elements, frequently several micrometers in length. As in the case of endogenous cellulose, the microfibril width of this newly synthesized cellulose is on the order of 2–3 nm. The in vitro cellulose microfibrils are bundled together to form small packets consisting of 3–10 units.

Fig. 4D illustrates an area of a sample as in Fig. 4C, but prior to Updegraff treatment. In this figure, a cellulose microfibril seems to be associated at one of its tips with a globular particle, which may correspond to a rosette-like structure that has survived the NaOH treatment used for the purification of the cellulose. The association of in vitro microfibrils with globular particles at one of their tips is also illustrated in Fig. 5, where a small fascicle of cellulose microfibrils is seen originating from a globular mass (arrow). Remarkably, the other ends of these microfibrils can be identified as reducing ends, since they can be labeled with streptavidin-gold (arrowheads) following a biotinylation reaction.

Electron Diffraction Characterization—A low dose electron micrograph of randomly distributed unstained microfibrils of in vitro cellulose is shown in Fig. 6. The inserted powder diagram corresponds to the diffraction pattern of an area of the specimen 1 µm in diameter. In this diagram, three rings can be clearly observed, a very sharp one at 0.2585 nm and two broader ones centered at 0.43 and 0.40 nm. Other rings close to the central beam may be present but cannot be visualized,
Fraction pattern of an area of the specimen of 1/2/H9262 recorded without negative staining. The inset labeled as described by Imai et al. (42) with gold particles (arrowheads) revealing their reducing ends. Scale bar, 0.1 μm.

Since they are lost in the diffuse central halo originating from the inelastic scattering. Following an indexation according to the monoclinic unit cell of cellulose I given by Sugiyama et al. (46), the ring at 0.2585 nm is unambiguously assigned to the meridional 004 reflection. Its sharpness indicates that the cellulose crystals have a high perfection along the chain direction. The two rings at 0.43 and 0.40 nm can be assigned to the 012 and 200 reflections of cellulose. These rings are somewhat wider than the 004 ring. Their larger width is expected from the line-broadening phenomenon resulting from the diffraction of cellulose crystals that are very long in the chain direction but substantially narrower in a perpendicular direction.

X-ray Diffraction—A series of diffraction diagrams was collected for each sample. The diagrams were averaged and corrected for background. Ten representative diagrams were used for the endogenous and bacterial celluloses, whereas the average trace of the in vitro sample was deduced from the superposition of 20 diagrams. The diffraction data are presented in Fig. 7, which shows the 20 scans obtained by azimuthal integration of the averaged diagrams of the three specimens. In Fig. 7, the inset is a composition that reproduces 120° sectors of the three corresponding powder diffraction patterns. Sector A corresponds to the in vitro sample, sector B to the endogenous sample, and sector C to the bacterial cellulose standard. A visual comparison of these patterns indicates marked differences in the crystalline features of the three samples.

The traces of the three diagrams clearly show the strongest cellulose reflections, namely 110, 110, 012, 200, and 004. Depending on the specimen, some of these reflections appear either individual or merged into one another. All three diagrams display the sharp meridional 004 diffraction peak at a d-spacing of 0.2585 nm (dashed vertical line). Besides this common feature, the three diagrams are substantially different. The bottom diagram corresponds to the in vitro sample. In this diagram, in addition to the sharp 004 peak, one notices two broad peaks: a very strong one centered at 0.40 nm and another one of a lower intensity at 0.55 nm. The middle diagram was obtained from endogenous cellulose; its strongest peak is slightly less intense and broader than the one corresponding to the in vitro sample. In addition, when comparing the traces of the endogenous and in vitro samples, the strongest peak has slightly moved toward longer d-spacings, since it is centered at 0.41 nm in the endogenous sample as opposed to 0.40 nm for the in vitro specimen. The second most intense peak at 0.55 nm is roughly at the same position in both patterns. It is nevertheless slightly broader and more intense for the endogenous sample than for the in vitro cellulose. The top trace in Fig. 7 is that of bacterial cellulose. This diagram contains sharp peaks due to the high crystallinity of the sample. The reflection 200 is the strongest. It is located at 0.396 nm (i.e., at a d-spacing significantly lower than those observed for the other two diagrams). In the case of bacterial cellulose, the other peaks, namely 110 (0.54 nm) and 110 (0.60 nm), are clearly resolved, whereas the peak corresponding to the 102 reflection (0.43 nm) occurs as a shoulder in the left foot of the 200 peak.

Table II gives some quantitative data for the characteristic crystalline parameters of the three samples. The in vitro cellulose presents a significant higher crystallinity and larger lateral crystal size than the corresponding endogenous polymer.
these observations, we can speculate that enzymes from various plant species may have a different lipid environment and, consequently, that the selection of the detergent used to extract the cellulose synthase complexes from a given species is critical. Also, it could well be that the enzyme complexes are located in the plasma membrane within microdomains that have a specific lipid composition. If this is the case, the detergents that allow in vitro synthesis of cellulose may not actually solubilize the enzymes but extract them as intact complexes together with structural lipids required for the cohesion of the multimeric synthases.

It seems that the in vitro synthesis of cellulose can be achieved only when intact enzyme complexes containing all of the proteins required for cellulose polymerization and spinning of the chains into microfibrils have been extracted. This idea is further supported by the observation, at the tips of nascent microfibrils, of globular particles that have the same appearance as rosettes. The presence of similar particles at the ends of the in vitro microfibrillar products was also reported by Kudlicka and Brown (14). In addition, the fact that the in vitro microfibrils have a diameter in the same range as their endogenous counterpart suggests that it is the very same rosettes that are responsible for endogenous or in vitro cellulose synthesis.

Interestingly, the globular particles that may correspond to the synthesizing enzyme complexes appear to be attached to the nonreducing ends of the cellulose molecules (Fig. 5). This result is in keeping with the work of Koyama et al. (55), where the addition of glucose units on the cellulose microfibrils from Acetobacter aceti was proven to occur at the nonreducing ends of the growing ribbons. The question of the direction of chain growth remains controversial, however, since cellulose chains from A. xylinum were described by other authors to elongate from the reducing ends (56). Interestingly, it has been demonstrated that β-chitin microfibrils, the counterpart of cellulose I in the chitin world, are polymerized from their nonreducing ends (57). It is therefore likely that polymerization of both cellulose I and β-chitin chains occurs in a similar way (i.e. from their nonreducing ends).

In their work on the in vitro synthesis of β-glucans from the cotton fiber, Kudlicka et al. (13) have defined conditions that favor cellulose biosynthesis. These imply the presence of MgCl$_2$ (8 mM) and CaCl$_2$ (1 mM) in the reaction mixture. Bivalent cations were also recently described by Peng et al. (16) to be required for cellulose synthesis. The results from these authors showed that Mops buffer favors the synthesis of Updegraff-sensitive and -resistant (1→4)-β-glucans over (1→3)-β-glucan. Similarly, in our work Mops proved to be the best buffer for in vitro synthesis of cellulose. However, as opposed to the data reported for the cotton fiber (13, 15, 16), the addition of bivalent cations was not always necessary for cellulose synthesis from R. fruticosus cell-free extracts. Indeed, the highest yield of in vitro cellulose was obtained with taurocholate extracts incubated in the absence of Mg$^{2+}$ and Ca$^{2+}$. The presence of Mg$^{2+}$ was necessary, however, to obtain the highest cellulose synthase activity from Brij 58 extracts. Mg$^{2+}$ and Ca$^{2+}$ are believed to be involved in a coregulation process of the cellulose and cellulose synthases or, more directly, in the catalytic activity of both enzymes. However, their role in the mechanisms of β-glucan synthesis remains to be clarified.

A sitosterol-β-glucoside was identified as a primer for cellulose synthesis in the cotton fiber (16). The addition of this kind of compound in our reaction mixture was not necessary for in vitro synthesis of cellulose. It is likely that this primer, if required for cellulose synthesis in R. fruticosus, is present in the preparations obtained after detergent extraction. It is also possible that the in vitro synthesis of cellulose does not start de
In Vitro Synthesis of Cellulose by Plant Cell-Free Extracts

novo in our preparations but that elongation of the chains rather occurs from preexisting cellulose molecules or chain fragments that are too small to be identified by TEM. If this is the case, the addition of a primer such as the sitosterol-β-glucoside (16) is not necessary for a successful in vitro synthesis of cellulose.

Our in vitro reactions have allowed the synthesis of enough cellulose to perform biochemical, chemical, and ultrastructural characterizations but also to probe the sample by an x-ray analysis that revealed some of the specific characteristics of the in vitro cellulose. The analysis of the diffraction diagrams of the in vitro and endogenous celluloses indicates that the two polymers can be classified as cellulose IV (58) (i.e. a variety of cellulose I). As for cellulose I, a fiber diagram of cellulose IV displays a sharp meridional reflection at 0.2585 nm, indicating a quasiperfect organization along the microfibril length. Perpendicular to this direction, the diagram of cellulose IV contains only two broad equatorial diffraction rings near 0.39 and 0.55 nm, as opposed to the diagram of cellulose I, which displays three sharper equatorial rings at 0.40, 0.54, and 0.60 nm. Cellulose IV may be considered as a disorganized state of cellulose I occurring when the fibrillar sample contains crystalline domains that are too narrow or too disorganized to be considered as real cellulose I crystals. Cellulose IV occurs in nature and has been observed in plant primary walls and fungal cell walls (58–64). In these cases, the diameter of the microfibrils is such that they contain no more than 20–40 cellulose chains. Of these, only 30–50% correspond to the core of the crystalline microfibrils, the remaining being at the surface. With such a small number of chains, the definition of “crystal” no longer applies, and a given microfibril is better defined as a loose arrangement of parallel cellulose chains held together by a nonperiodic system of hydrogen bonds, with the net result of very poor lateral coherence, despite a nearly perfect longitudinal organization. The result of this situation is the occurrence of diffraction diagrams as in Fig. 7, where the equatorial 200 reflection is close to the position of the corresponding reflection of cellulose I but where the peaks 110 and 110 have collapsed into one reflection (65).

The comparison of the diffraction diagrams of the endogenous and in vitro celluloses shows significant differences between the two samples. The calculated lateral crystal size of the in vitro sample is larger than that of the endogenous sample. In addition, the position of the 200 diffraction peak in the endogenous sample is shifted toward higher d-spacings by 0.01 nm with respect to that of the in vitro sample. This shift is small but nevertheless meaningful. Indeed, several x-ray analyses of cellulose samples that were systematically decrystallized showed that the creation of defects induces a shift of the 200 diffraction peak toward higher d-spacings, indicating some swelling of the cellulose unit cell along its a axis (48, 66, 67). In light of these data, a comparison of traces A and B in Fig. 7 indicates clearly that, despite identical microfibrillar widths, the endogenous cellulose is less perfect than the in vitro sample. These structural differences are in agreement with the observed difference in sensitivity toward the Updegraff reagent. This acid treatment chops the endogenous cellulose microfibrils into smaller elements, whereas it leaves the in vitro microfibrils nearly intact. The drastic effect of the Updegraff reagent on the endogenous cellulose and its limited action on the in vitro sample is also reflected in the difference in DPn inferred from the methylation analysis of both samples. Whereas the Updegraff treatment had reduced the DPn of the endogenous sample, it is far less damaging for the in vitro specimen. Indeed, as opposed to the case of endogenous microfibrils, the methylation analysis of the Updegraff-treated in vitro product did not yield any 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl glucitol (Fig. 2). These data allow us to conclude that the DPn of the corresponding sample was high, since 100 is considered to be the lower detection limit of the method.

Reasons to account for the difference in crystallinity and chemical sensitivity of the two samples can be proposed. Despite their morphological similarity, the in vitro and endogenous cellulose microfibrils differ markedly in their state of aggregation. In the wall of R. fruticosus cultured cells, as in other plant primary walls, the cellulose microfibrils are organized in more or less interwoven fascicles (63, 68). Thus, during their deposition, the microfibrils need to push their way through the already laid compact cell wall, with the result of a number of defects, the occurrence of the ubiquitous microfibril-twists being the most prominent (69). It seems reasonable to state that, in contrast to the endogenous cellulose, the in vitro microfibrils are able to grow without restraint in a neighbor-free environment and contain fewer defects. This hypothesis is well supported by the differences in the two specimens, either in terms of their crystallinity or their sensitivity toward the Updegraff reagent.

For the first time, our data open new opportunities to isolate and characterize directly the machinery responsible for cellulose biosynthesis. Another benefit of the present study was to show that the in vitro cellulose microfibrils had specific structural superiority (and therefore higher mechanical strength) by comparison with the cellulose from primary walls. These properties together with the insensitivity of these microfibrils toward acid hydrolysis are quite attractive for potential applications in the field of material science, in particular those of composites and nanocomposites (70). In this context, the fact that the in vitro microfibrils occur as dispersed aqueous suspensions make them a unique system in the world of cellulose. Indeed, for various industrial applications, considerable energy is being spent to disrupt cellulosic structures in order to disperse their microfibrils (71, 72). As seen here, such dispersions are readily achieved during in vitro experiments. It remains, however, to devise conditions to produce large quantities of in vitro cellulose in the absence of callose before exploiting fully the potential of these preparations.

Acknowledgments—We thank Dr. C. Riekel from the European Synchrotron Research Facility (Grenoble, France) for the use of beam line ID13, Prof. G. B. Fincher and Dr. M. Hrmova (University of Adelaide, Australia) for the generous gift of the (1→3)-β-D-glucan endohydrolase from barley, and M.-F. Marais (CERMAV, Grenoble, France) for technical assistance with plant cell cultures and methylation analysis.

REFERENCES

1. Nikaido, H., and Hassid, W. Z. (1971) Adv. Carbohydr. Chem. Biochem. 26, 386–392
2. Northcote, D. H. (1991) in Bioisynthesis and Biodegradation of Cellulose (Haigler, C. F., and Weimer, P. J., eds) pp. 165–176, Marcel Dekker, New York
3. Delmer, D. P., and Amor, Y. (1995) Plant Cell 7, 987–1000
4. Brown, R. M., Jr. (1996) J. Macromol. Sci. Pure Appl. Chem. A33, 1345–1375
5. Delmer, D. P. (1999) Annu. Rev. Plant Physiol. Plant Mol. Biol. 50, 245–276
6. Turner, S. R., and Somerville, C. R. (1999) Cell 9, 689–701
7. Arioli, T., Peng, L., Betzner, A. S., Burn, J., Wittke, W., Horth, W., Camilleri, C., Höfte, H., Plazinski, J., Birch, R., Cork, A., Glover, J., Redmond, J., and Williamson, R. E. (1998) Science 279, 717–720
8. Taylor, N. G., Scheible, W. R., Cutler, S., Somerville, C. R., and Turner, S. R. (1999) Plant Cell 11, 769–779
9. Fagard, M., Desnos, T., Desprez, T., Goubet, F., Refregier, G., Mouille, G., McCann, G., Rayon, C., Vernettes, S., and Höfte, H. (2000) Plant Cell 12, 2409–2423
10. Taylor, N. G., Laurie, S., and Turner, S. R. (2000) Plant Cell 12, 2529–2539
11. Okuda, K., Li, L., Kudlicka, K., Kuga, S., and Brown, R. M., Jr. (1993) Plant Physiol. 101, 1101–1114
12. Amor, Y., Haigler, C. H., Johnson, S., Wainscott, M., and Delmer, D. P. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 9353–9357
13. Kudlicka, K., Brown, R. M., Jr., Li, L., Lee, J. H., Shin, H., and Kuga, S. (1996) Plant Physiol. 107, 111–1123
14. Kudlicka, K., and Brown, R. M., Jr. (1997) Plant Physiol. 115, 643–656
15. Kudlicka, K., Lee, J. H., and Brown, R. M., Jr. (1996) Am. J. Bot. 83, 274–284
16. Peng, L., Kawage, Y., Hogan, P., and Delmer, D. (2002) Science 295, 147–150
