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Note

Silver labeling of the reducing ends of bacterial cellulose

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The unidirectional ("parallel") orientation of glucan molecules in crystalline native cellulose (cellulose I) has been claimed to be highly likely by studies of X-ray diffraction and molecular packing analyses based on data with *Valonia* cellulose[1,2]. Although the conclusion has been taken almost as proven, partly because of the likeliness of the structure from the biochemical point of view, independent and more direct evidence has been sought.

Recently, such evidence has been obtained through electron microscopical studies. Hieta *et al.*[3] labeled with silver the reducing ends of cellulose exposed at the tip of a fragmented microfibril of *Valonia* and showed an asymmetrical distribution of reducing ends, which indicated a unidirectional alignment of glucan chains. The same feature was visualized also by Chanzy and Henrissat[4], who showed that the digestion of *Valonia* cellulose by cellobiohydrolase proceeds in one direction in a single microfibril.

Although there are good reasons to believe that the crystal structure of native cellulose is unique, the parallel-chain structure of celluloses in other organisms has still to be confirmed. The earlier attempt[3] to apply the silver staining technique to ramie cellulose was unsuccessful. In this study, we have stained the reducing ends of bacterial cellulose ribbons, which have a morphology intermediate between those of algal and higher plant celluloses.

Fig. 1 shows the typical appearance of fragments of bacterial cellulose labeled with silver by the two-step staining procedure (see Experimental). Many of these fragments are labeled at the tips with dark spots ~5 nm in size which are obviously silver particles. The staining is apparently asymmetrical, thus indicating that glucan chains in the microfibril are oriented with the same polarity. It is known from the earlier study[5] that *Acetobacter* secretes 50–80 cellulose microfibrils 3.0–3.5 nm in

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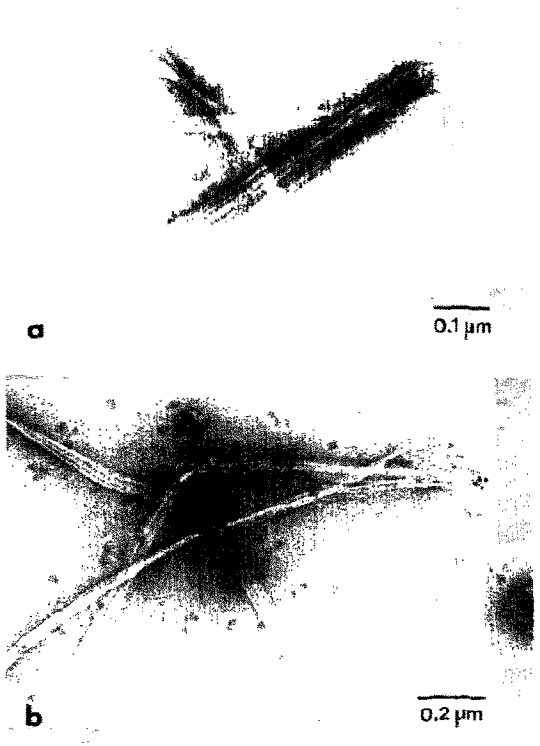

Fig. 1. Fragments of bacterial cellulose ribbon labeled with silver at the reducing ends. The asymmetrical staining demonstrates that the cellulose molecules are arranged in the same sense in the microfibrils. Silver is deposited on silver proteinate particles which are attached to the reducing end of cellulose.

width, which are subsequently aggregated into flat ribbons. The fragments in Fig. 1 seem to originate from single ribbons produced by single cells.

Fig. 2 is a low-magnification micrograph of the same specimen, which demonstrates the ubiquity of the asymmetrical staining. The feature is not dependent on the length of fragments, and many long fragments could be traced to a labeled "head" and a non-labeled "tail". The specimen also contained many large bundles of microfibrils, which obviously originate from bundles of ribbons formed in the pellicle. Some of these bundles were found to be stained at one end and others at both ends, as in Fig. 3. By closely examining such bundles, the unit of asymmetrically stained ribbon fragments can be distinguished. This situation is likely to be the result of random fasciation of the ribbons, which are produced by randomly oriented bacterial cells during cellulose synthesis.

Although nothing is known about the direction of synthesis of cellulose, it seems reasonable to assume that the β-D-glucose residue is linked (1→4) to the non-reducing end of the preceding chain, as with glycogen[6], so that the ribbons in

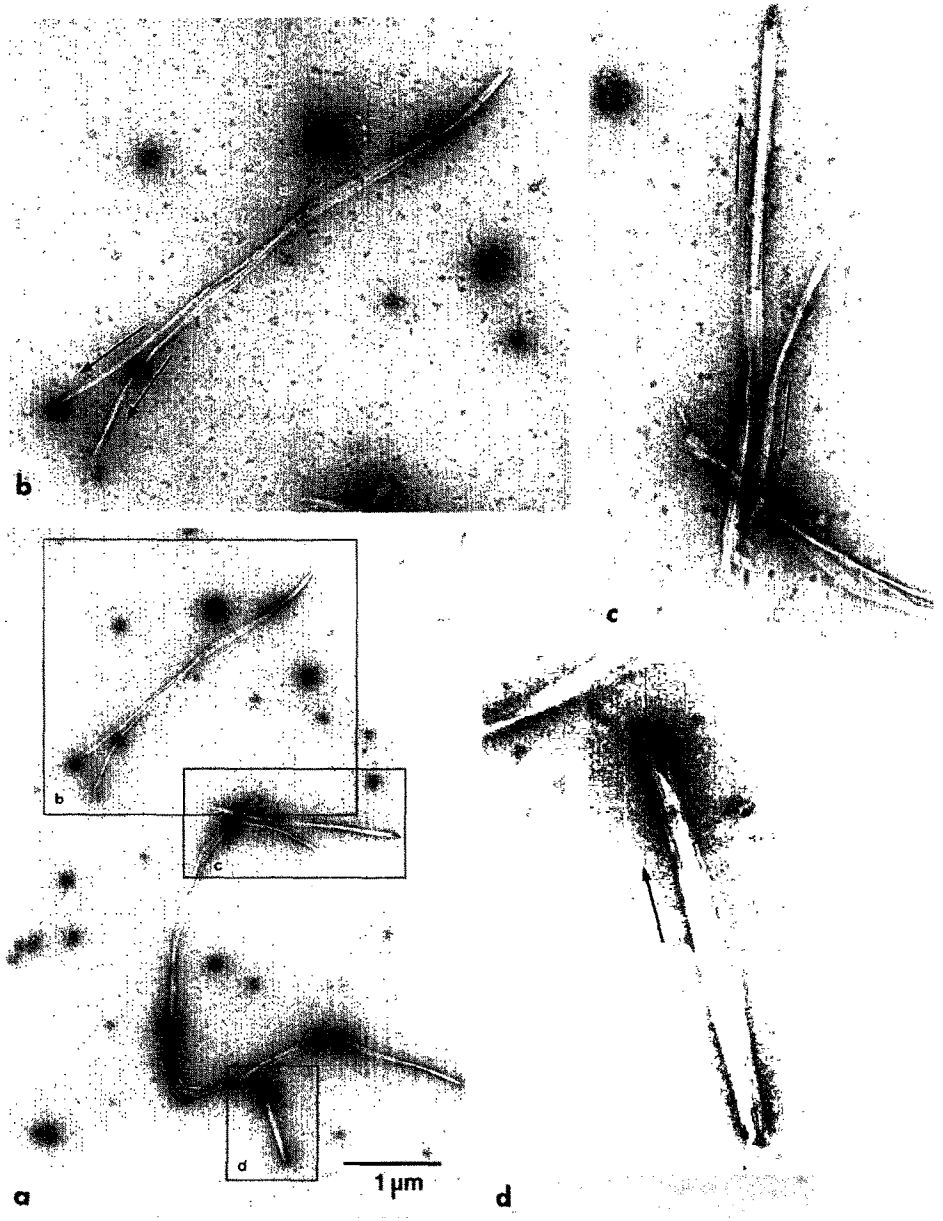

Fig. 2. (a) Low-magnification version of Fig. 1; (b)–(d) higher magnification of the framed areas in (a), showing asymmetrical staining. Arrows point towards the stained ends.

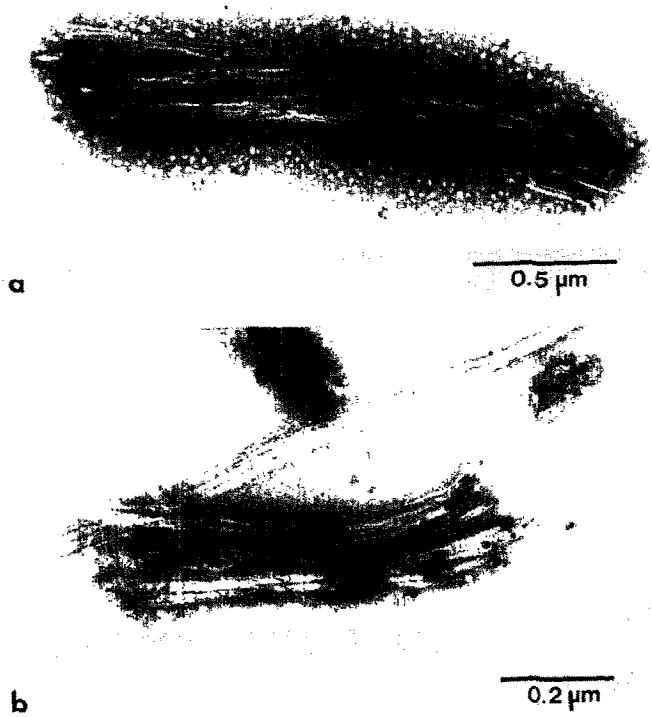

Fig. 3. Fragments from multi-ribbon aggregates in the same specimen as in Fig. 1: (a) mostly aligned in a same sense, (b) the middle part is aligned opposite to other parts.

Fig. 4. Microfibril fragments of *Valonia* cellulose stained by the same method as in Fig. 1.

Fig. 2 should have been produced in the direction of arrows (the bacterial cell traveling to the opposite).

The present technique of silver proteinate-nucleated silver staining seems to be more specific and efficient for the staining of the reducing end than the previous method[3] applied to *Valonia* cellulose. When this material was stained by the present technique, asymmetrical labeling resulted (Fig. 4).

Thus, the parallel-chain structure is confirmed with two different sources of native cellulose. The structure is consistent with the extended chain structure visualized by high-resolution electron microscopy[7,8]. It also corroborates the scheme of unidirectional synthesis of cellulose by a single type of enzyme, which is organized in the membrane-associated complex called "terminal complex", and almost immediate fasciation into crystalline microfibrils (see, for example, ref. 9).

EXPERIMENTAL

Cellulose sample. — *Acetobacter xylinum* (American Type Culture Collection, No. 23769) was incubated in Schramm and Hestrin's medium[10] for 1 week. The resulting gel-like cellulose pellicle was thoroughly washed with deionized water (used throughout), treated with boiling aqueous 2% sodium lauryl sulfate for 1 h (in order to remove bacterial cells), and washed with water. The wet material was crushed in liquid nitrogen with a porcelain mortar and pestle, then treated with 2M HCl for 30 min at 100°, and washed with water by centrifugation (4000*g*, 15 min). The treatment with acid cleaved the cellulose ribbons at mechanically damaged regions, exposing the reducing or non-reducing end groups at the tips. For comparison, a similar sample was prepared from the cell wall of *Valonia macrophysa*.

Silver staining. — The first step was based on thiosemicarbazide–silver proteinate (TSC-SP) labeling[11]. A suspension of cellulose sample (1 mL) was mixed with 1% thiosemicarbazide (TSC) in aqueous 5% acetic acid. The mixture was kept for 90 min at 60° and then washed by centrifugation. The sample was resuspended in water (1 mL) and mixed with aqueous 1% silver proteinate (SP, Merck) in aqueous 2% sodium borate (5 mL) [this solution was freshly prepared and filtered through a 0.45-μm membrane filter (Milex HA, Millipore Ltd.)]. The mixture was kept for 1 h in the dark and then diluted with water for centrifugation.

The second step is necessary for the visualization of the reducing ends because the amount of silver in bound SP was not sufficient to be observed with the electron microscope. SP is considered to provide the nuclei for silver deposition. A suspension (1 mL) of the TSC-SP-treated sample suspension was mixed with silver–ammonia solution (5 mL, see below) in a nitric acid-cleaned glass vial. The mixture was kept for 3–6 min at 95°, until the suspended particles turned brown. The hot mixture was then diluted quickly with water (100 mL), and the sample was washed with water by centrifugation.

Silver–ammonia solution was prepared by dissolving silver nitrate (1 g) in water (7.5 mL), and adding conc. NH_3 (0.8 mL). The brown precipitate was

dissolved by further dropwise addition of conc. NH_3. The solution was then diluted to 75 mL and filtered through a 0.45-μm membrane. This solution can be stored for several weeks in the dark, but occasional filtration (preferably immediately before use) is desirable.

Electron microscopy. — The sample was negatively stained on a carbon-coated Formvar grid with aqueous 2% uranyl acetate (containing 0.1% of bacitracin as a wetting agent) and examined with Philips 420 (80 kV) or Hitachi H-800 (75 kV) instruments.

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