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On the polarity of cellulose in the cell wall of *Valonia*

Junji Sugiyama[1]*, Henri Chanzy[1], Jean François Revol[2]

[1] Centre de Recherches sur les Macromolécules Végétales CNRS-CERMAV, affiliated with the Joseph Fourier University of Grenoble, B.P. 53X, F-38041 Grenoble Cedex, France
[2] Pulp and Paper Research Institute of Canada, McGill Pulp and Paper Research Centre, 3420 University Street, Montréal, Québec, H3A 2A7, Canada

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Abstract. The orientation of the triclinic phase of cellulose in the cell wall of *Valonia ventricosa* J. Agardh was investigated by X-ray- and electron-diffraction analysis. In addition to the well-documented uniplanar-axial organization of the cell wall which requires that the a^* axis should be always perpendicular to the wall surface, the direction of this axis was also found to be pointing outward from the plasma membrane side of the wall. This unidirectionality was persistent throughout the various layers that constitute the cell wall and also for the three microfibrillar orientations that occur in *Valonia* cell walls. The unidirectionality of the a^* axis indicates, in particular, that the *Valonia* cellulose microfibrils are not twisted along their axis. These observations are consistent with a cellulose biosynthetic scheme where a close association exists between terminal-complex orientations and those of the cellulose microfibrils. In this context, the unidirectionality of the a^* axis of cellulose seems to be related to the restricted mobility of the terminal complexes which are able to slide in the plasma membrane but not to rotate along their long axis.

Key words: Cellulose crystal – Cellulose microfibril synthesis – Cellulose polymorphism – Triclinic cellulose – *Valonia*

Introduction

The crystalline organization of native cellulose has been the subject of a number of X-ray investigations during the last 60 years. So far, these studies have failed to provide a unified crystallographic model which would satisfy all the cellulose specimens that are encountered in nature. From the analysis of ^{13}C solid-state nuclear magnetic resonance (NMR) spectroscopy data, it was proposed that in native cellulose two crystalline forms, cellulose Iα and Iβ, may coexist (Atalla and VanderHart 1984; VanderHart and Atalla 1984). This idea led to the re-examination of the crystallography of cellulose by Sugiyama et al. (1990, 1991). These authors confirmed that the electron-diffraction fiber pattern of the highly crystalline *Valonia* cellulose could be fully indexed by describing the specimens in terms of a mixture of two crystalline phases (Sugiyama et al. 1990). Subsequently, these two phases were clearly identified by electron-microdiffraction techniques in *Microdictyon*, another alga whose cell wall also contains highly crystalline cellulose (Sugiyama et al. 1991). One crystallographic phase (the Iα form) corresponded to a one-chain triclinic unit cell with parameters $a = 0.674$ nm, $b = 0.593$ nm, c (chain axis) $= 1.036$ nm, $\alpha = 117°$, $\beta = 113°$, $\gamma = 81°$. The other phase (the Iβ form) was attributed to a two-chain monoclinic unit cell with parameters $a = 0.801$ nm, $b = 0.817$ nm, c (chain axis) $= 1.036$ nm, γ (the monoclinic angle) $= 97.3°$.

The cell wall of *Valonia* consists of a superposition of successive layers, each of them containing a parallel array of cellulose microfibrils (Preston 1974). As schematically drawn in Fig. 1, three orientations of these microfibrils alternate from one layer to the next to produce a criss-crossed structure (Preston 1974). A mature cell contains up to 60 layers each of about 3–5 microfibrils thick (Goto et al. 1973; Revol 1982). The *Valonia* cell wall presents a characteristic uniplanar orientation (Preston 1974; Tanaka and Okamura 1977); two of the four faces of the microfibrils, that are square in cross section (Revol 1982), are preferentially parallel to the cell wall surface. They correspond to the planes having a 0.6-nm d-spacing. The other two faces, identified as the planes having a 0.53-nm d-spacing, are perpendicular to the cell wall. For the triclinic component, which is dominant in *Valonia* cellulose, this uniplanar orientation means that the a^* reciprocal axis is always perpendicular to the cell wall surface.

The uniplanar orientation has proven to be crucial in some aspects of the crystallography of *Valonia*. It has

* *Permanent address*: Wood Research Institute, Kyoto University, Uji, Kyoto 611, Japan

Abbreviation: TC = terminal complex

Correspondence to: H. Chanzy; FAX: (33)-76547203

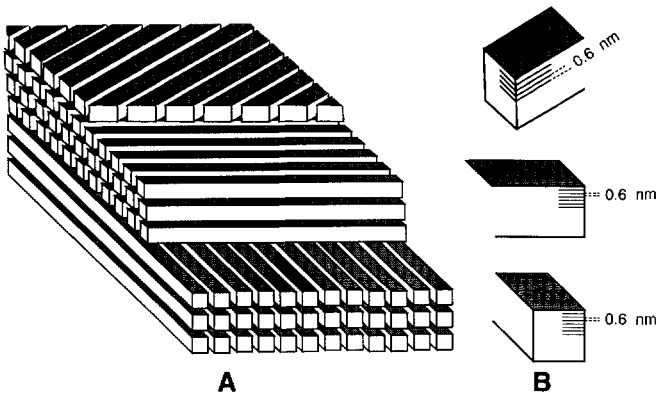

Fig. 1A–B. **A** Schematic representation of the organization of the cell wall of *Valonia*. Cellulose microfibrils having square sections are organized along three sets of orientation: two major sets consisting of three to five layers of cellulose microfibrils and a minor set consisting of only one to two layers of cellulose microfibrils. The two major sets have microfibrillar directions that are nearly perpendicular whereas in the minor set the direction is nearly at 45° with respect to those of the major sets. **B** Within each layer, the microfibrils are organized in such a way that their 0.6-nm planes: ($\bar{1}10$) in the monoclinic phase and (100) in the triclinic phase, are parallel to the wall surface

allowed the detection of the two opposite "up" and "down" directions of the fibre *c*-axis. This detection was done by X-ray diffraction on the whole cell wall (Preston 1974, Tanaka and Okamura 1977), by electron microdiffraction performed on ultrathin sections for the individual layers (Revol and Goring 1983) and confirmed by lattice imaging (Sugiyama et al. 1985). It is again this uniplanar orientation that revealed the triclinic symmetry of *Valonia* cellulose. Indeed for a monoclinic crystal with *c* as monoclinic axis, $Fhkl = F\bar{h}k\bar{l} = Fhk\bar{l} = F\bar{h}\bar{k}l$ and, therefore, the four quadrants of a diffraction diagram containing c^* should be equivalent. On the other hand, for a triclinic crystal, Fhkl is no longer equivalent to $F\bar{h}\bar{k}l$. Thus, a dissymmetry results between the left and the right part of the corresponding diagram. Such dissymmetric pattern will be maintained when the diffraction is from a bundle of parallel cellulose crystals only if a certain degree of register exists among them. This situation is the case for the *Valonia* cell wall, where the dissymmetry was first reported by Honjo and Watanabe (1958); they were thus able to prove by electron diffraction that *Valonia* cellulose had a triclinic character.

Since, as a result of the uniplanar orientation in *Valonia*, the a^* reciprocal axis of the triclinic phase is perpendicular to the cell wall, an important question is raised concerning the directionality of this a^* axis. Indeed, it may be pointing outward or toward the plasma membrane or be distributed in both directions. In real space, these two possibilities mean that the *a* axis of the triclinic phase may point above or below the cell wall surface or be distributed in both directions. The knowledge of this directionality would help in understanding the occurrence of the uniplanar orientation in *Valonia*, which may result purely from an intrinsic property of the cellulose microfibrils or be directly related to their biogenesis. The present work addresses this problem by analyzing the triclinic a^* directionality in each layer of the *Valonia* cell wall by means of electron diffraction and in the whole cell wall by X-ray diffraction.

Materials and methods

Valonia ventricosa J. Agardh vesicles were harvested from the sea bed in the Lower Keys, Fla., USA. The fresh cells were slit open and their walls allowed to dry by pressing them between two sheets of filter paper. The dried walls were scraped with tweezers to remove most of the non-cellulosic components. The cell walls were then boiled three times for 3 h in distilled water and then three times for 2 h in 0.1 N NaOH under nitrogen. The purified cell walls were then rinsed with distilled water, neutralized with dilute HCl, washed again with distilled water until neutrality, and dried between two sheets of filter paper.

Electron-diffraction analysis. Fragments of *Valonia* cell wall were delaminated with sharp needles and mounted on carbon-coated grids. Specimens having only one layer of microfibrils were analyzed with a model EM 400T electron microscope (Philips, Eindhoven, The Netherlands) operating at 120 kV. Thicker specimens, consisting of several layers were analyzed with a Philips CM 30 operating at 300 kV. All diffraction diagrams were recorded on Mitsubishi electron-image plates that were developed in Kodak D19 developer.

X-Ray analysis. All experiments were done using a Philips PW 1720 X-ray generator operated with nickel-filtered Cu-Kα radiation. Fragments of purified *Valonia* cell wall were examined with a stereomicroscope under polarized light. Domains where the two main microfibrillar directions cross almost at right angles were chosen for X-ray analysis. For this, the selected wall area was mounted on a 0.020-inch X-ray collimator in such a way that its plasma-membrane side was toward the recording film (Fig. 2). The X-ray diagrams were first recorded with the sample flat, and the two main fiber axes, as well as the third minor one, were identified. A series of diagrams were then obtained after orienting the cell wall fragments to bring into Bragg position the two $\bar{1}03$ diffraction spots corresponding to the two main fiber directions. To achieve these orientations, the two azimuthal directions $+26°$ and $-26°$ were selected off the two main fiber axes, with the result that four directions were successively oriented vertically. For each direction, an X-ray diagram was recorded after rotating the specimen by 14° about a horizontal axis. From these four diagrams, only two displayed the expected $\bar{1}03$ diffraction spot along the vertical axis.

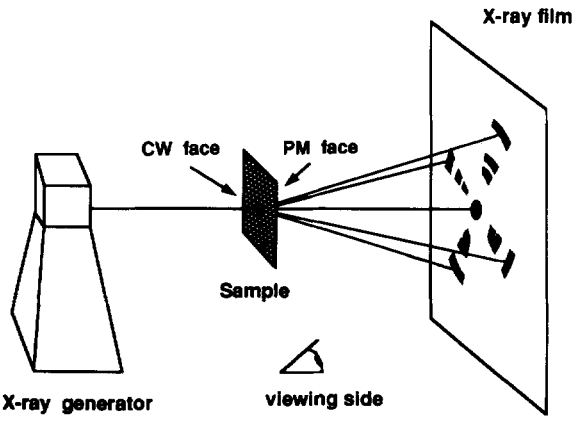

Fig. 2. Schematic diagram showing the geometry of the *Valonia* cell wall with respect to the X-ray generator and the X-ray film. *CW*, cell wall face: facing the exterior of the cell; *PM*, plasma membrane face: facing the interior of the cell

Results

Theoretical diffraction diagrams of Valonia cellulose. Given the aforementioned data on the orientation of *Valonia* cellulose, there are four independent ways in which a triclinic cellulose microfibril can be laid within a uniplanar cell wall layer of *Valonia*, as opposed to two for the monoclinic case. These six modes are schematically presented in Figs. 3A (triclinic) and 3B (monoclinic). In Fig. 3A, the four triclinic microfibrils (1–4) correspond to the two directions for *c* combined with the two directions for *a**. In Fig. 3B, only two independent monoclinic microfibrils (5, 6) need to be considered; they correspond to the two directions for *c*.

Figure 4A–C represents the three different diffraction diagrams that are expected when the diffraction is performed on an individual layer of *Valonia* cell wall. These diagrams can be correlated to the six possible situations shown in Fig. 3A,B. The diagram in Fig. 4A is expected for microfibrils belonging to situations 1 or 3. On the other hand, the diagram in Fig. 4B corresponds to either situation 2 or 4. The diagram in Fig. 4C is unique for the monoclinic case and corresponds to microfibrils in either of situations 5 or 6.

Experimental diffraction diagrams. When one layer of parallel microfibrils of *Valonia* cellulose is examined by electron-diffraction analysis (Fig. 5A), the corresponding dia-

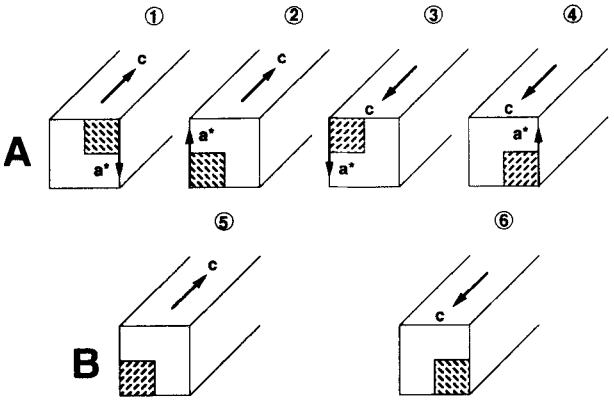

Fig. 3A,B. The six possibilities that can be distinguished by diffraction analysis for laying a *Valonia* cellulose microfibril within a uniplanar cell wall layer while keeping the *a** axis vertical. **A** The triclinic case, situations 1–4; **B** the monoclinic case, situations 5 and 6

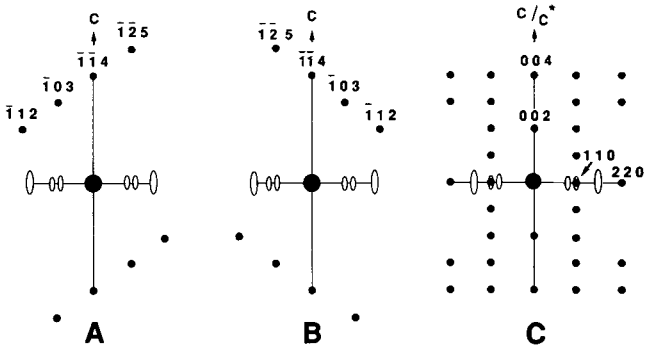

Fig. 4A–C. A, B The two types of diffraction diagram resulting from a triclinic cellulose crystal with its *c* axis vertical and containing the 1̄03 diffraction spots. **A** corresponds to crystals as in Fig. 3A, situations 1 and 3; **B** corresponds to crystals as in Fig. 3A, situations 2 and 4. **C** The diffraction diagram resulting from a monoclinic cellulose crystal with its *c*/*c** axis vertical and containing the 110 diffraction spot (see Fig. 3B, situations 5 and 6). For clarity, the strong equatorial diffraction of cellulose has been indicated by *white ellipses* in the figures

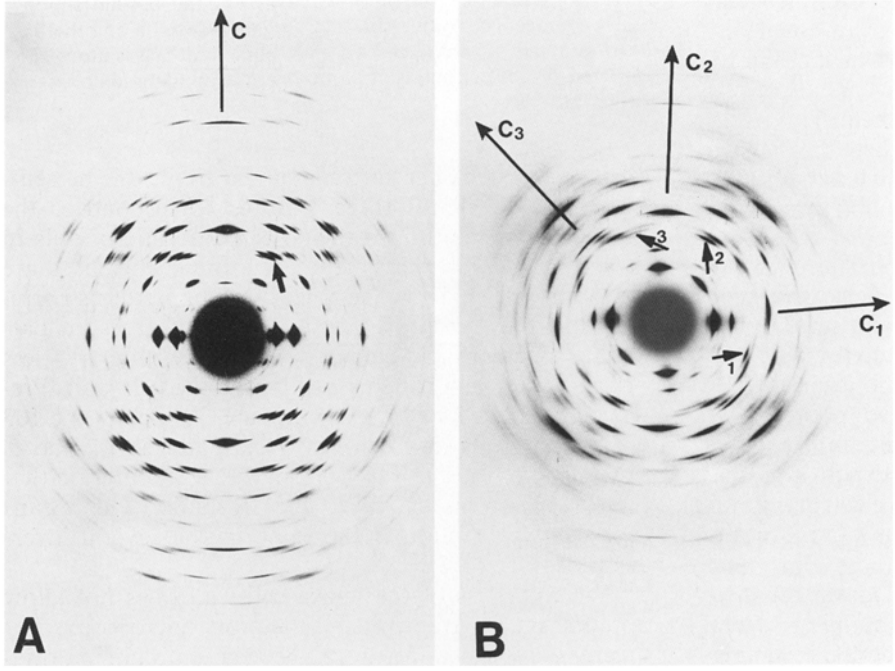

Fig. 5. A Electron-diffraction diagram of one layer of parallel microfibrils from *Valonia* cellulose with the *c* axis vertical. The diffraction spot indicated by the *small arrow* corresponds to the 1̄03 reflection that is not mirrored in the four quadrants of the diagram. **B** Electron-diffraction diagram of a thick specimen of a *Valonia* cell wall recorded at an acceleration voltage of 300 kV. The three microfibrillar orientations are marked as c_1, c_2 and c_3. The *small arrows* 1, 2 and 3 correspond to the three 1̄03 reflections corresponding to the three orientations

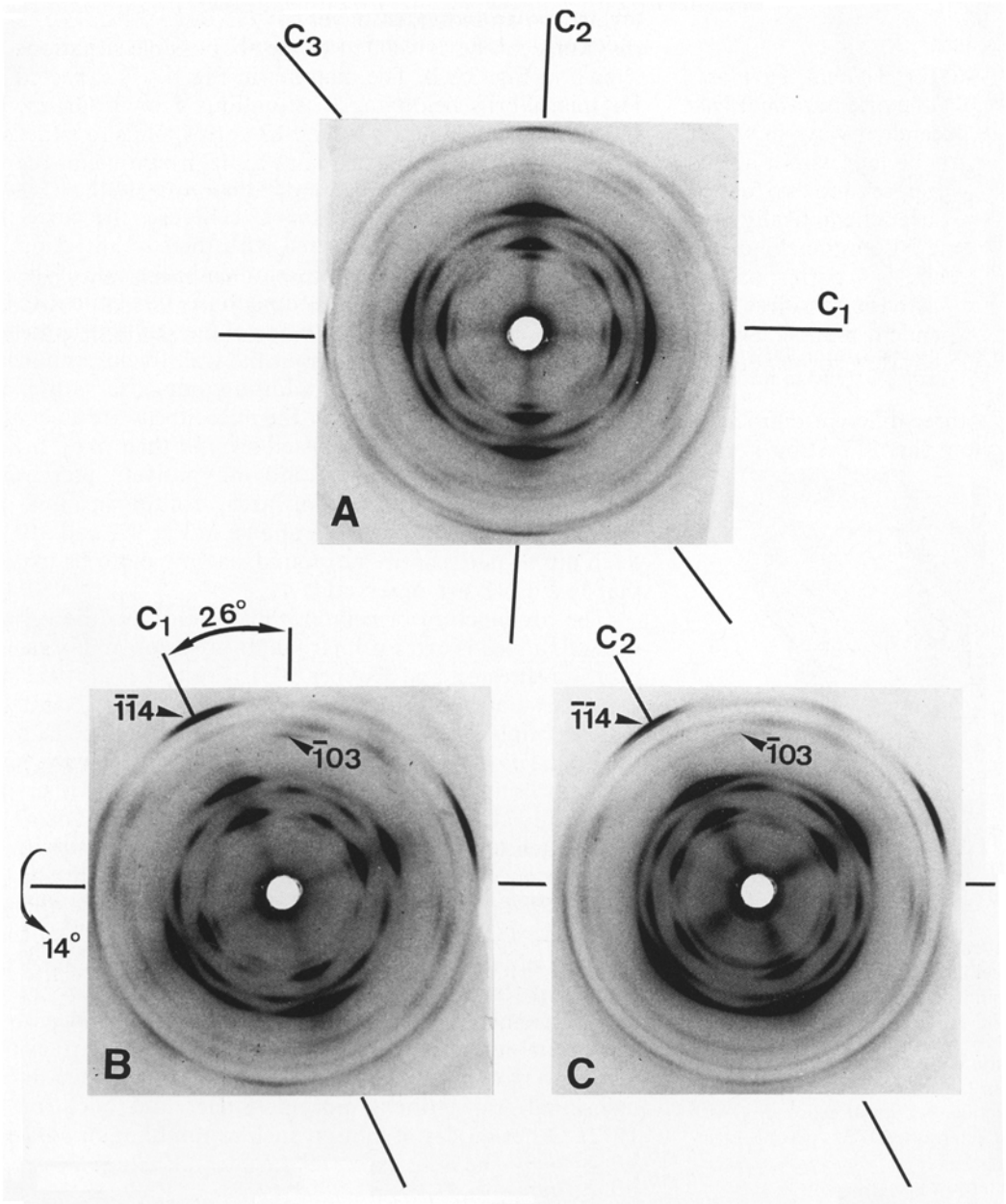

Fig. 6A–C. **A** Typical X-ray diffractogram of a fragment of *Valonia* cell wall oriented as in Fig. 1. The three fibrillar directions of the wall are marked as c_1, c_2 and c_3. **B** Same as in **A** but rotated by 26° in order to bring to the vertical the azimuthal direction corresponding to the 103 diffraction line of the part of the diagram having c_1 as fiber axis. The specimen was then rotated around a horizontal axis by 14° before recording the pattern. **C** Same as in **B** but for the part of the diagram having c_2 as fiber axis. In both **B** and **C**, the $\bar{1}\bar{1}4$ diffraction line, indexed along the triclinic unit cell is along the corresponding fiber axis

gram displays several diffraction spots that are not mirrored in the four quadrants of the diagram. Typically, this dissymmetry occurs on the first and third layer line, and the diffraction spot at 0.306 nm, identified as $\bar{1}03$ (arrowed in Fig. 5A) is representative of such a phenomenon. The diagram in Fig. 5A contains information about the 4B type. Therefore, the corresponding specimen has triclinic domains that correspond exclusively to situations 2 or 4, indicating that the a^* axes point only in one direction with respect to the plasma-membrane side of the cell wall. Obviously, if the specimen grid was turned upside down, the corresponding electron diffractogram would contain information of the 4A and not of the 4B type.

Figure 5B shows an electron diffractogram recorded at 300 kV from a thicker specimen containing several layers of a *Valonia* cell wall. This diffractogram is a superposition of three fiber patterns similar to the one presented in Fig. 5A. These three patterns correspond to the three major orientations of the cellulose microfibrils in the cell wall. For convenience, the three fiber axes are indicated in Fig. 5B as c_1, c_2 and c_3. The patterns corresponding to c_1 and c_2 are rather strong whereas the pattern corresponding to c_3 is weaker, reflecting this less frequent microfibrillar orientation in the cell wall (Preston 1974). The small arrows 1,2 and 3 point to the $\bar{1}03$ diffraction spots that belong to the patterns 1,2 and 3, respectively. Thus, it is clear from this diffractogram that the three patterns belong to the 3B family. This means that the a^* direction is the same in the several layers examined in Fig. 5B.

The thickness of the whole cell wall does not allow observation by transmission electron microscopy. For this reason X-ray diffraction is needed to obtain informa-

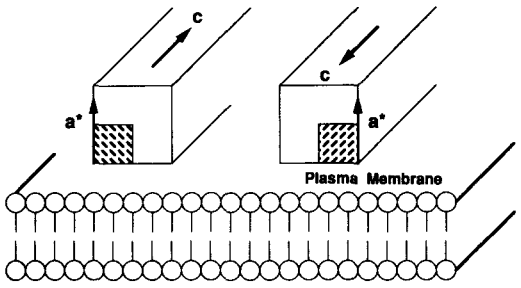

Fig. 7. The two possibilities for the orientation of the triclinic components of *Valonia* cellulose with respect to the plasma membrane. In both cases, the a^* axis is pointing outward from the plasma membrane side of the cell wall and the c axis is parallel to the plane of the plasma membrane

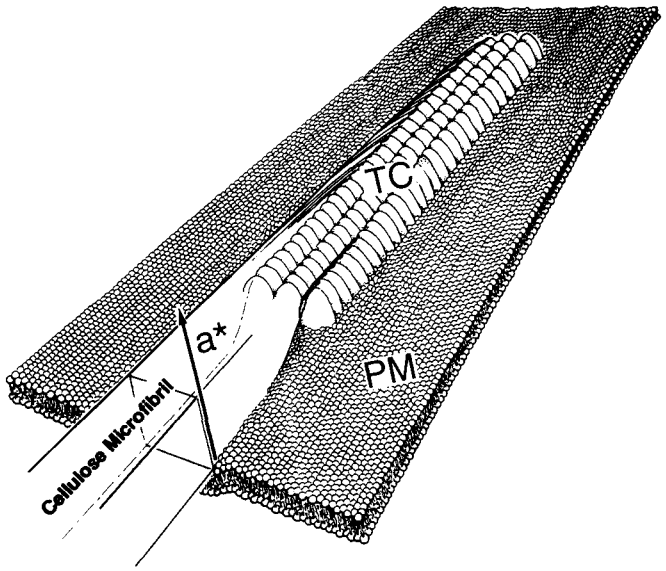

Fig. 8. Model (adapted from Itoh and Brown 1984) of a *Valonia* microfibril assembling in association with a TC. The a^* axis of the triclinic component of the microfibril is vertical and upward. The c axis is along the microfibril direction but its directionality with respect to the TC remains to be determined. *PM*, plasma membrane; *TC*, terminal complex

tion on the crystalline structure of whole *Valonia* cell wall fragments. Figure 6A shows an X-ray diffractogram corresponding to a specimen in which the plasma-membrane side of the cell wall faces the X-ray film (Fig. 2). As in Fig. 5B, the three main orientations of the *Valonia* cell wall are clearly identified and the three corresponding c axes outlined. The triclinic character of the patterns is revealed by orienting the specimens successively about the four directions described in the *Materials and methods*. From these four directions, only two gave patterns in which the $\bar{1}03$ spots could be detected as shown in Figs. 6B and 6C. In both cases, the $\bar{1}03$ spot occurs on the right-hand side of the corresponding fiber axis. Therefore, in the two main fibrillar orientations the a^* of cellulose triclinic crystals always points outward from the plasma membrane. This experiment cannot be done for the third minor fibrillar orientation since the corresponding $\bar{1}03$ diffraction spot is hidden behind the diffraction spots of the two stronger patterns.

Discussion

The results presented in this study demonstrate that, for the triclinic component of *Valonia* cellulose, the corresponding crystals are organized with their a^* direction pointing outward from the plasma-membrane side of the cell wall (Figs. 7, 8). This phenomenon is not limited to one layer of microfibrils, nor to one of the sets of orientation, but is persistent throughout the wall thickness and for the three orientations. In addition, our observations indicate that within each layer the microfibrils are essentially laid flat and are not twisted around their axes. Indeed, twisted microfibrils would alternatively present their a^* axis upwards and downwards, resulting in a mixture of diagrams such as those shown in Fig. 4A and 4B. Such mixed patterns are not found, as only patterns like that in Fig. 4B are observed.

The architecture of cellulose microfibrils in the cell wall of *Valonia* is extraordinary in its precision and organization (Preston and Kuyper 1951; Preston et al. 1953; Cronshaw and Preston 1958; Preston 1974). Indeed, with the exception of the first-synthesized cellulosic lamella, a growing *Valonia* cell maintains the ability to lay down microfibrils in three specific directions and no others (Preston 1974). At the surface of the *Valonia* plasma membrane, each cellulose microfibril was shown to be in close association with a terminal complex (TC; Itoh and Brown 1984). By freeze etching, these authors were able to observe TCs with lengths up to 600 nm (average length 350 nm), not only on the outer leaflet of the plasma membrane (EF face) but also on the interior leaflet of the plasma membrane (PF face). According to the fluid mosaic model for cell membranes, the *Valonia* TCs are expected to have a freedom restricted essentially to two-dimensional translational motion (Singer and Nicolson 1972). Other modes of motion such as tumbling or rotation around the long axes of the TCs are unlikely. Thus, we can tentatively correlate the absence of twisting in *Valonia* microfibrils with the restriction in the degree of freedom of the TCs, on the one hand, and their close association with the emerging microfibrils, on the other.

Knowledge of the orientation of the a^* axis with respect to the plasma membrane is not sufficient to determine completely the orientation of the cellulose unit cell with respect to the TC (Fig. 8). In order to know whether the c axis is pointing towards or outward from the corresponding TC, one would need to obtain electron-diffraction patterns and corresponding images of cellulose microfibrils attached to their synthesizing TCs. So far, this observation has not been possible as the TCs invariably deteriorate when they are extracted from their living environment. This lack of data explains why only one of the unit cell axes of cellulose can be drawn with certainty in Fig. 8.

Our diffraction experiments are informative only for the triclinic component of *Valonia* cellulose. The monoclinic domains which account for almost 40% of the total

sample (Debzi et al. 1991) are crystallized in a more symmetrical way. In such domains, the data resulting from diffraction experiments are unable to differentiate between the two directionalities of a^*. In particular, it is impossible to detect by electron diffraction whether the cellulose microfibrils were twisted in the monoclinic domains. However, since it has been demonstrated in one case that the triclinic and the monoclinic domains alternate along the same microfibrils (Sugiyama et al. 1990), it is likely that the crystalline orientation of cellulose is maintained in both domains. It remains to be explained why two crystallographic phases occur during the biosynthesis of such perfect cellulosic cell walls as in *Valonia*.

The above observations are so far limited to the *Valonia ventricosa* cell wall. For other green algae, such as *Chaetomorpha megalonium* (Nieduszinski and Atkins 1970) and *Microdictyon tenuius* (Sugiyama et al. 1991), the X-ray- and electron-diffraction patterns that were recorded perpendicular to the wall surface also clearly indicate a unidirectional character for the a^* axis of the triclinic phase. However, these X-ray patterns were not referenced with respect to the plasma membrane of the corresponding cells. It is therefore not possible at this stage to decide whether the above phenomenon occurs with these specimens. Also its universality in other green algae that display a *Valonia*-like type of lamellation should be established. Work is presently in progress to address this problem.

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