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ON THE CROSS-SECTIONAL SHAPE OF CELLULOSE CRYSTALLITES IN *VALONIA VENTRICOSA*

J.-F. REVOL

Pulp and Paper Research Institute of Canada, 570 St John's Boulevard, Pointe Claire, Quebec, Canada H9R 3J9

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ABSTRACT

The cross-sectional shape of the cellulose crystallites from the Valonia ventricosa *cell wall has been investigated by electron microscopy, using negative staining and Bragg contrast in the bright field mode, with emphasis on this latter technique. The appearance of the cellulose crystallites in the electron microscope depends on their orientation. The cross-section of each cellulose crystal is almost square, with an average side of 18 nm. Sub-units corresponding to elementary fibrils were not detectable within the crystals. The differences between these results and those of earlier workers are discussed.*

INTRODUCTION

Cellulose, the main constituent of plant cell walls exists as microfibrils of indefinite length. However, a controversy remains concerning the lateral size and shape in cross-section of the microfibrils. The confusion comes from the difference between the width of the structures observed through the electron microscope and the width of the cellulose crystallites deduced from X-ray diffraction. By electron microscopy, 3·5 nm elementary microfibrils were observed in different plants (Frey-Wyssling, 1969; Manley, 1964; Heyn, 1968) while by X-ray line broadening considerably larger crystallites were measured, with dimensions which depended on the plant species (Nieduszynski & Preston, 1970; Caulfield, 1971). Cellulose from green algae such as *Valonia* is known for its high degree of crystallinity and for its broad microfibrils (Preston, 1974). Frey-Wyssling *et al.* (1966) observed that when *Valonia* is ultrasonicated some areas along the microfibrils are disrupted into 3·5 nm elementary

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microfibrils. Gardner & Blackwell (1971) likewise observed regular fibrillar fragments of 3·5 nm or multiples thereof. On the other hand, Goto *et al.* (1978) using the same technique of ultrasonic disintegration found that the fibrillar fragments have a width distribution centred on 3 nm but with no tendency for the microfibrils to disrupt into any multiple of 3 nm. Using electron diffraction and Bragg contrast in the dark-field mode, Bourret *et al.* (1972) found cellulose crystallites in the cell wall of *Valonia ventricosa* with widths ranging from 14 to 18 nm without persistent 3·5 nm sub-units. Using cross-sections and negative staining, Goto *et al.* (1973) found in the cell wall of *Valonia macrophysa* cellulose crystallites having a rectangular cross-sectional shape, with the larger side of 15–20 nm parallel to the surface of the cell wall and the shorter side of 7–10 nm. This latter result is in line with the fact that cellulose microfibrils in *Valonia* would be relatively flat ribbons (Preston, 1974), and that the wider face, corresponding to the crystallographic plane $(2\bar{2}0)$,* would lie parallel to the surface of the cell wall (Preston, 1974; Tanaka & Okamura, 1977).

The purpose of the present work is to obtain more information on the cellulose crystallites of *Valonia ventricosa* and their arrangement in the cell wall. The cross-sectional shape and size of the crystallites were investigated using thin sections in conjunction with negative staining and Bragg contrast in bright field electron microscopy. It will be shown that the cross-section of the cellulose crystallites in *Valonia ventricosa* is more or less square in shape; this differs from the general findings of earlier workers (Preston, 1974; Goto *et al.*, 1973) which indicated a rectangular shape.

It may be noted that in the present paper, the terms cellulose crystallite and microfibril are used synonymously, since in native *Valonia ventricosa* the microfibrils are found to consist of long crystallites having widths corresponding exactly to that of the microfibrils (Bourret *et al.*, 1972; Lazaro & Chiaverina, 1973).

EXPERIMENTAL

Materials

Samples of *Valonia ventricosa* preserved in formaldehyde were purified successively in aqueous sodium hydroxide, distilled water, hydrochloric acid and distilled water as described by Gardner & Blackwell (1971).

Sample Preparation

A *Valonia* cell wall was cut into pieces suitable for X-ray diffraction. The patterns obtained with the X-ray beam perpendicular to the cell wall allowed recognition of the two main directions of the cellulose microfibrils (Preston, 1974). These small pieces of *Valonia* were then soaked for 2 days in water and dehydrated by solvent exchange with ethanol, followed by propylene oxide and embedded in epoxy resin (Epon 812).

* Throughout this paper, the indexing of native cellulose refers to the 8-chain unit cell as proposed by Gardner & Blackwell (1974), with *C* as the fibre axis.

Sections, approximately 50–100 nm thick and transverse with respect to one of the two directions of the microfibrils, were made with a diamond knife mounted on an ultramicrotome (Porter Blum). The sections were collected on carbon coated grids and examined in the electron microscope. Some preparations were treated with a solution of potassium hydroxide in propylene oxide and methanol in order to remove the epoxy resin (Maxwell, 1978) and were negatively stained with uranyl acetate.

Electron Microscopy

A transmission electron microscope (Philips EM400T) was used. The accelerating voltage was 80 kV for normal imaging and 120 kV for Bragg contrast imaging. For this latter technique, a thin foil objective aperture of 20 μm diameter was used. In such a condition all the electrons diffracted by the planes in the Bragg position are outside the aperture and stopped. Thus, the corresponding diffracting zones are absent from the bright field image. In order to maintain the crystallinity of the specimen during observation, the microscope was operated in the low intensity beam condition by overfocusing the first condenser lens.

The wavelength of electrons accelerated at 120 kV is 0·00335 nm. As a consequence of this short wavelength, the Bragg angle is less than 1° for the main (*hk*0) planes of the cellulose (equatorial planes). In addition, it has been shown recently (Roche & Chanzy, 1981) that an electron diffracting crystal of *Valonia macrophysa* goes out of the Bragg position after a deviation of 2°. As a consequence, the Bragg position will be satisfied when a set of planes is approximately parallel to the electron beam (vertical direction).

RESULTS

A section of the *Valonia ventricosa* cell wall with one of its two main fibrillar directions approximately perpendicular to the plane of the section is presented in Fig. 1. The embedding resin has been removed and the preparation negatively stained with uranyl acetate. In such a condition, only the crystallites are not stained and are seen as white objects on the micrograph. When white ribbons are visualised, the cellulose crystallites are longitudinal or oblique with respect to the plane of the section. On the other hand, when white dots are present, the cellulose crystallites are approximately transverse. Accordingly, a succession of lamellae parallel to the cell wall surface and containing either longitudinal or vertical cellulose microfibrils can be seen in Fig. 1. The width of each lamella corresponds to several layers of parallel microfibrils. However, an irregular orientation of the microfibrils in each lamella is often noticed, probably due to a disruption that occurs during the removal of the resin. Figure 2 shows a lamella presenting its microfibrils approximately transverse to the section. The white dots can be seen with more or less sharp contours, depending on the tilt angle between the microfibrils and the electron beam, as already discussed by Goto

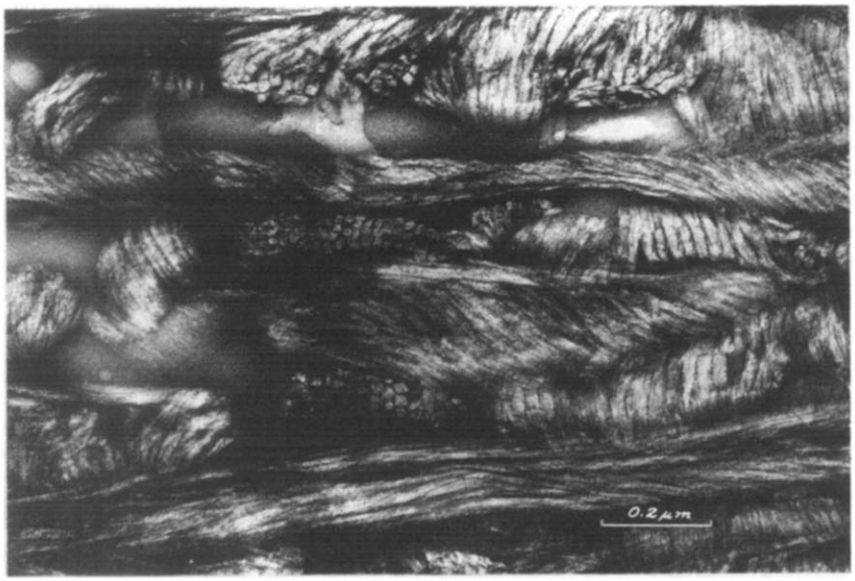

Fig. 1. Electron micrograph of a transverse ultrathin section of *Valonia ventricosa* cell wall. The epoxy resin (Epon 812) has been removed and the preparation negatively stained with uranyl acetate. The cell wall surface is parallel to the horizontal of the picture.

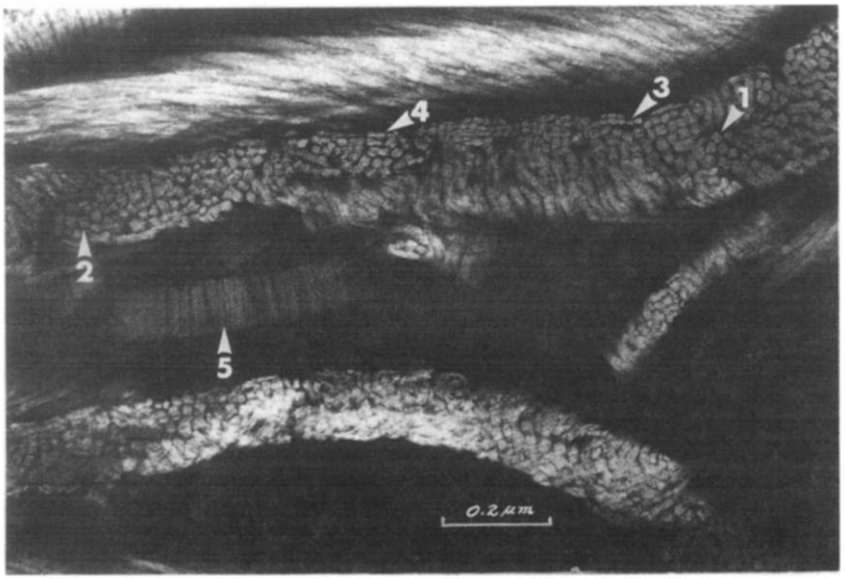

Fig. 2. As Fig. 1, but another area.

et al. (1973) with *Valonia macrophysa*. When the contours are sharply defined, this means that the microfibrils should be vertical and the white dots represent their cross-sectional shape. This shape appears square or near to square, with size ranging from 10 to 20 nm, with a higher frequency at 18 nm. Most of the well defined cross-sectioned microfibrils present this squarish shape (see arrows 1 and 2 in Fig. 2). Very often the presence of crystallites in registry can be observed as shown by arrows 3 and 4, where rows of crystallites having their faces parallel to each other are present. One of the faces of these crystallites is preferentially parallel to the cell wall surface. Sometimes some layers of microfibrils which have been cross-sectioned lie parallel to the supporting film showing the real thickness of the section (see arrow 5) which is 80 nm in this case.

To have a better representation of the true arrangement of the cellulose crystallites in the cell wall as well as the shape of the crystallites themselves, sections were observed without removing the resin and without any staining, using Bragg contrast in the bright field mode. In this mode, the crystallites having crystallographic planes in the Bragg position with respect to the electron beam, i.e. approximately parallel to the electron beam, are seen as black areas on the micrograph. Figure 3 presents a typical micrograph of a *Valonia ventricosa* cell wall section using such a technique. In contrast to Fig. 1, the structure of the cell wall seems well protected in its matrix of

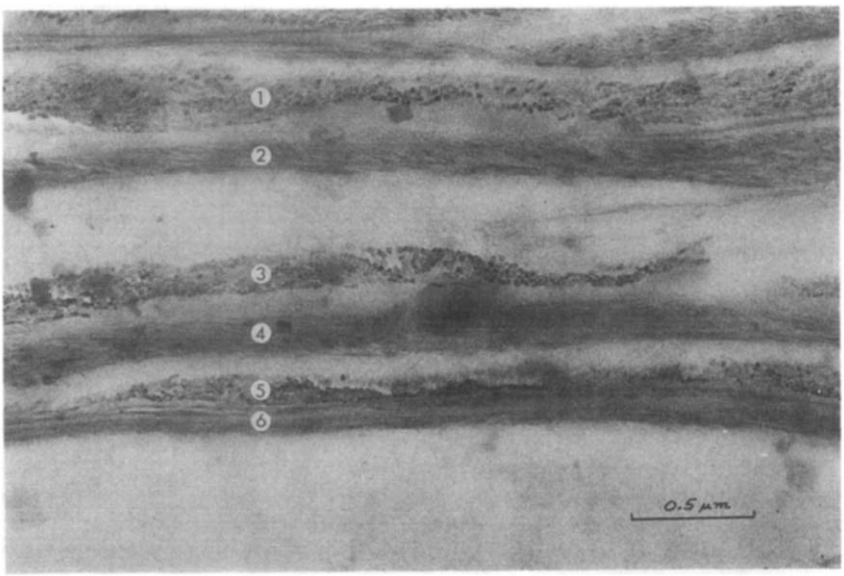

Fig. 3. Bright field images slightly underfocused in the Bragg contrast mode of a transverse ultra-thin section of *Valonia ventricosa* cell wall embedded in epoxy resin (Epon 812). The Bragg contrast has been obtained by using a 20 μm thin foil objective aperture. The cell wall surface is parallel to the horizontal of the picture.

epoxy resin, but some lamellae are often separated, indicating that the cell wall is easily delaminated. On the other hand, it is hardly possible to see layers or monolayers of parallel microfibrils separated from a given lamella. In Fig. 3, the image has been obtained using a slightly underfocus condition which produces a phase contrast effect. This phase contrast applied to the polymers (Roche & Thomas, 1981) allows enhancement of the boundaries between crystalline zones (the cellulose crystallites) and amorphous zones (the resin matrix). As a consequence, even the cellulose crystallites which were not in the Bragg position can be visualised. In Fig. 3, the microfibrils in the lamellae 2, 4 and 6 contain no area in the Bragg position (no black areas are clearly visible) but they are visualised as long bands darker than the epoxy resin, indicating that they are long microfibrils running approximately parallel to the surface of the film support. On the other hand, the lamellae 1, 3 and 5 mainly contain microfibrils in a vertical position or nearly so. Instead of long and dark bands, the lamellae appear to be composed of dots, most of which are black, indicating that a high percentage of crystalline zones are in the Bragg position. Since it is known that the fibre axis (*C*-axis) of the crystallographic unit cell is parallel to the microfibril axis (Preston, 1974), the equatorial planes are all parallel to the microfibril axis and of course will be in the diffracting condition when the cellulose crystallite is vertical; this explains the high density of diffracting zones in such a condition. Most of the clear and sharp black dots are square or roughly square (see Fig. 4(a)) and represent

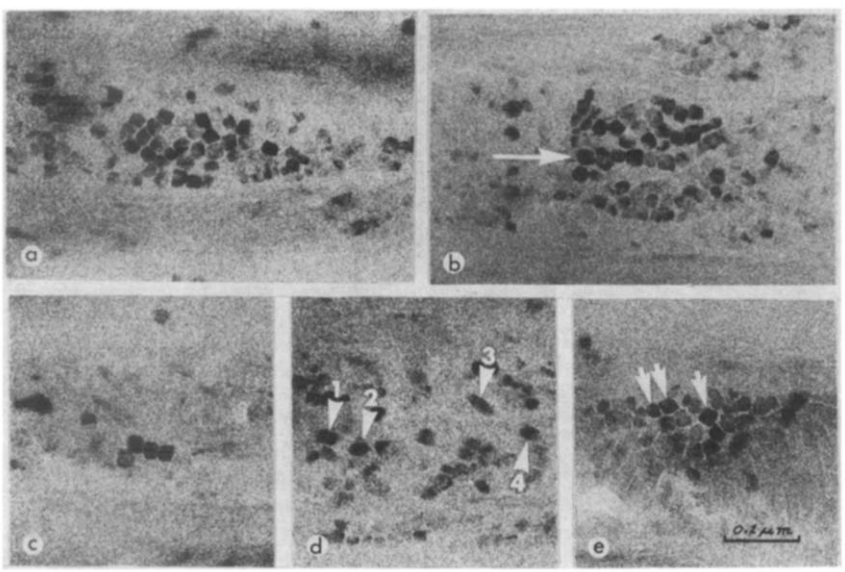

Fig. 4(a–e). As Fig. 3, but other areas and enlarged.

the cross-sectional shape of the cellulose microfibrils. The size is again the same as found by negative staining, with a high frequency at 18 nm. Some rows of microfibrils aligned parallel to the surface of the cell wall are visible as shown by the arrow in Fig. 4(b). While some black dots are nearly perfect squares (see Fig. 4(c)), some others are rather oval or rectangular with not so well defined contours (see Fig. 4(d)); this will be considered in the discussion.

DISCUSSION

In the present work, the structure of the cell wall found in *Valonia ventricosa* confirms that it is a 'crossed fibrillar' wall structure which is basically a two lamella-repeat with an approximately perpendicular fibrillar orientation (Preston, 1974). A consequence of this 'crossed fibrillar' structure is the facility to separate two successive lamellae, while inside a given lamella the fibrillar structure seems to maintain its original close packing. Even when epoxy resin was removed, only a few monolayers or fragments of monolayers (see arrow 5 in Fig. 2) were disrupted from their original lamella. This is an indication that one lamella contains parallel microfibrils tightly bonded together while the bonding between two adjacent lamellae is much weaker. Mention should be made here that a uniplanar orientation exists in *Valonia* with the $(2\bar{2}0)$ plane preferentially parallel to the surface of the lamellae (Preston, 1974; Tanaka & Okamura, 1977). Thus, a correlation must exist between this preferential orientation and the strong bonding system between the faces of the microfibrils (probably hydrogen bonding), the one being the consequence of the other.

The cross-sectional view of the cellulose crystallites in *Valonia ventricosa* shows that the shape is not always well defined but is mostly square or nearly square, in contrast with the work done by Goto *et al.* (1973) who found in *Valonia macrophysa* a rectangular shape with the larger face parallel to the cell wall surface. In interpreting their results, they considered the importance of the verticality of the microfibrils. This is also an important consideration when applying the Bragg contrast technique, as in the present paper. How tilted must one crystallite be from its vertical position to go completely out of its Bragg position? Suppose that θ is the angle between one equatorial plane and the plane of tilt (this plane is perpendicular to the tilt axis), and α is the tilt angle from the vertical position. The resultant angle β between the vertical (electron beam) and the equatorial plane can be readily derived and is given by the formula:

$$\sin\beta = \sin\alpha \sin\theta \tag{1}$$

We have seen in the experimental part, that a deviation of 2° is enough to exceed the Bragg condition (Roche & Chanzy, 1981). Let us assume that the maximum value of β allowing this equatorial plane to remain in the diffracting condition, is approximately

2°. It results in a maximum value α_m for the tilt angle α in a direction θ, given by the following relationship:

$$\sin \alpha_m = \frac{\sin 2^\circ}{\sin \theta} \tag{2}$$

Suppose now that a crystallite is in vertical position represented by the segment $0A = L$ in Fig. 5. The fibre axis C is parallel to the axis of the crystallite and one equatorial plane $(hk0)$ intersects the plane of observation along the $(hk0)$ direction. If the crystallite is tilted at an angle α_m in the direction θ, the segment $0A = L$ becomes $0A_1$ and its projection on the plane of observation is $0A_2 = 0A_1 \sin \alpha_m = L \sin \alpha_m$. The distance between the point A_2 and the line $(hk0)$ is $A_2A_3 = 0A_2 \sin \theta = L \sin \alpha_m \sin \theta$ and according to eqn (2) $A_2A_3 = L \sin 2^\circ = \text{constant}$.

A representation of the Bragg positions for the three equatorial planes (220), (2$\bar{2}$0), and (040) with respect to $L \sin \alpha$ (which is the projection of the crystallite on the plane of observation) and the direction of tilt θ is given in Fig. 6. For clarity, the planes are oriented as for an orthorhombic unit cell. The zones corresponding to the orientations of the crystallite for which the Bragg position of a given equatorial plane is maintained are well visualised (dark zones). Consider what happens when the crystallite is tilted along the direction $0C'$. The projection of the crystallite ($L \sin \alpha$) will be always on the line $0C'$. $0A$ represents this projection when the crystallite is tilted at an angle α_m corresponding to the maximum tilt angle before the plane (220)

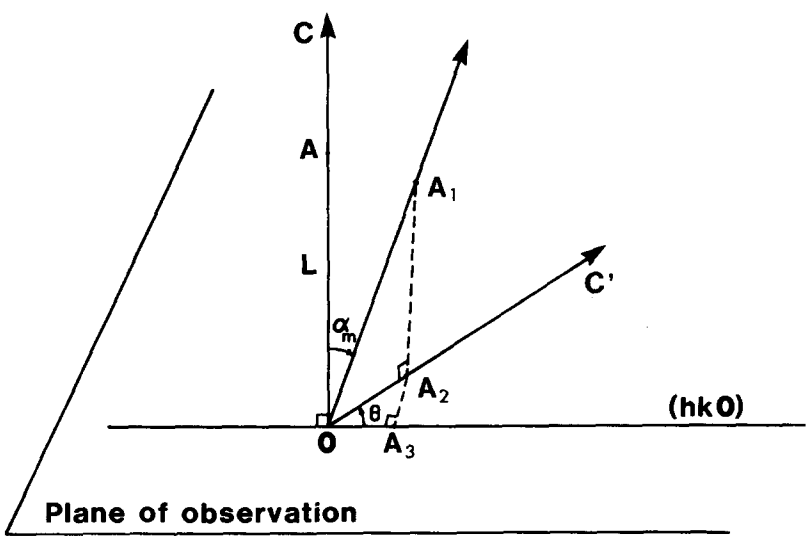

Fig. 5. Schematic representation of the projection on the plane of observation of one cellulose crystallite tilted at an angle α_m with respect to the vertical in a direction θ. The crystallite is represented by the segment $0A = L$.

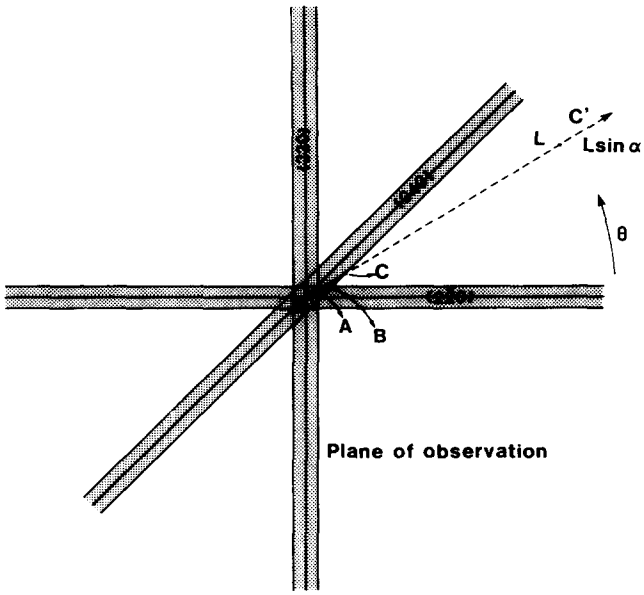

Fig. 6. Schematic representation of the Bragg positions for the equatorial planes of a cellulose crystallite. The crystallite is assumed to be a segment of length L as in Fig. 5. In the vertical position, the projection of the crystallite on the plane of observation will be the point 0. For a tilt angle α in any θ direction (tilt direction), the value of this projection will be $L \sin \alpha$. The dark zones correspond to the zones where the Bragg position is maintained for at least one equatorial plane. For more details, see the explanations in the text.

goes out of the Bragg condition. Similarly, $0B$ corresponds to the maximum tilt angle for the plane $(2\bar{2}0)$ and $0C$ for the plane (040). Thus, if the projection is between 0 and A, the Bragg condition is satisfied for the three planes, between A and B for the planes $(2\bar{2}0)$ and (040), and between B and C for the plane (040) only. Finally, beyond C the Bragg condition is no longer satisfied for these planes. When $\alpha = 90°$, the projected crystallite $L \sin \alpha$ becomes L (it is therefore in the plane of observation). As we are mainly interested in the crystallites near to their vertical positions (in order to interpret their cross-sectional shape), an enlargement of the central part, corresponding to small values of the tilt angle α, is seen in Fig. 7. The inner circle corresponding to a value of $\alpha = 2{\cdot}8°$ represents the area where at least one of the equatorial planes is in the Bragg position. The larger circle corresponds to a value of $\alpha = 4{\cdot}7°$ and shows that in quadrants I and III the Bragg condition is always maintained while in quadrants II and IV some area between $\alpha = 2{\cdot}8°$ and $\alpha = 4{\cdot}7°$ corresponds to a situation where the Bragg orientation is never satisfied (clear zones). For a tilt angle of $\alpha = 4{\cdot}7°$, it can be seen that the chance to keep a Bragg orientation, depending on the direction θ, is 75%. Finally, if the tilt angle α is greater than $4{\cdot}7°$, the only possibilities of remaining in the

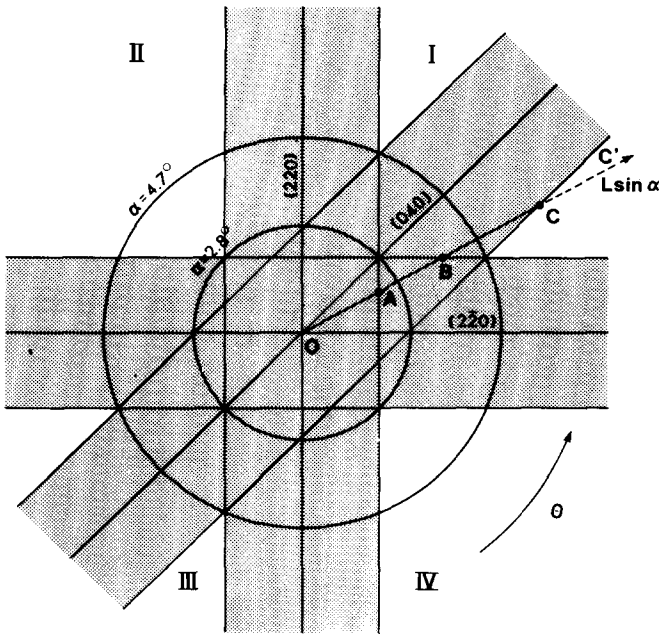

Fig. 7. Inner part of Fig. 6 corresponding to small values of the tilt angle α.

Bragg condition are to be found in a direction θ corresponding approximately to one of the equatorial planes.

Now, let us consider the effect on the cross-sectional view of one crystallite tilted in such a way that the Bragg condition is satisfied, as described above. Figure 8 represents the cross-section of a cellulose crystallite as a perfect square. The planes (220) and (2$\bar{2}$0) are parallel to the sides of the square, while the plane (040) is approximately along one diagonal. Suppose the length of the crystallite is 100 nm and the width of its lateral face 20 nm. Different situations depending on the θ direction and the angle of tilt α for which the crystallite is still in the Bragg position are represented. If the crystallite is tilted parallel to one side (case 1) this will produce a rather rectangular shape with the longer side well contrasted while the shorter side will be rather faded. If the crystallite is tilted along approximately one diagonal (case 2) this will deform the original square depending on the value of the tilt angle α to produce a rather oval shape, the two extremities being not so well defined. These two examples can be seen very often. The arrow 1 in Fig. 4(d) shows a rectangular shape while the arrows 2, 3 and 4 show oval shapes. Finally, cases 3 and 4 represent the deformation of the original square for a small value of the tilt angle and are mostly encountered, as seen by arrows in Fig. 4(e). However, it should be mentioned here that the real situation could be much more complex, because the shape of each crystallite may not be

exactly the same nor well defined, and because the crystallite is certainly not perfectly oriented all along its fibre axis. It could be either twisted around the fibre axis or undulated or both, with obvious consequences for the cross-sectional view. One of the best ways of knowing if a crystallite is vertical remains the sharpness of its contours. In addition, as seen in Figs 6 and 7, the probability of a crystallite being in the Bragg condition if the tilt angle α is larger than $4{\cdot}7^\circ$ is very low, in contrast with the high probability at smaller angles. So, the zones of high density of crystallites in the Bragg position as presented in Figs 4(a), 4(b) and 4(e) undoubtedly contain crystallites that are nearly vertical and the true cross-sections of these crystallites are close to the shapes observed through the electron microscope. Cases 3 and 4 of Fig. 8 demonstrate effectively that the deformation produced by such small tilt angles does not drastically change the original form, and in this schematic approach, a perfect square becomes a quasi square or quasi rectangle 10–30% larger in size than the original square.

In the light of these results, it seems improbable that the cellulose crystallites in the *Valonia ventricosa* cell wall have the pronounced rectangular cross-sectional shape found in *Valonia macrophysa* (Goto *et al.*, 1973). This raises a new and important question concerning the preferential orientation of the $(2\bar{2}0)$ plane parallel to the surface of the cell wall. If indeed the cellulose crystallites in *Valonia ventricosa* are mostly square in cross-section, then the preferential orientation cannot be explained

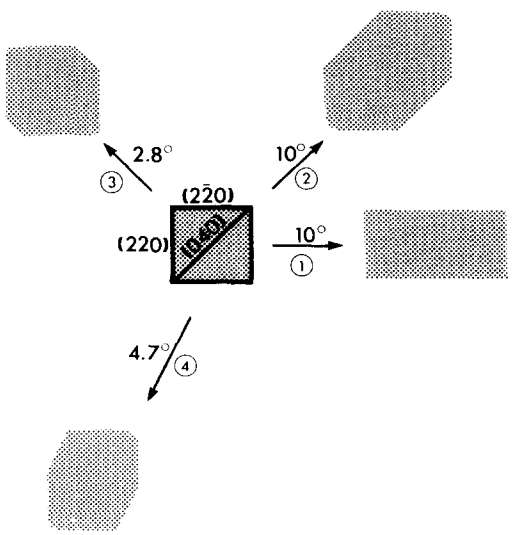

Fig. 8. Schematic representation of the resultant shape of a cross-section of one cellulose crystallite tilted in the direction indicated by the arrow. In the vertical position, the crystallite exhibits a perfectly square shaped cross-section having a 20 nm side. Its length is assumed to be 100 nm.

by their shape, and some other factor must govern their assembly in such a registry. At present, we can only speculate about the nature of this factor and the origin of the preferential orientation remains an open question.

Finally, it has not been possible to detect inside one cellulose crystal any sub-units corresponding to the elementary microfibrils observed after sonication (Frey-Wyssling *et al.*, 1966; Gardner & Blackwell, 1971). Thus, if such elementary microfibrils exist within the cellulose crystallite of *Valonia*, they must be packed in such perfect registry that for all practical purposes, they cease to exist as separate entities detectable by using the Bragg contrast imaging in our microscope.

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