Combined Infrared and Electron Diffraction Study of the Polymorphism of Native Celluloses

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ABSTRACT: In this study, 10 cellulose samples of various origins were subjected to heat annealing in 0.1 N NaOH at 260 °C. The initial and annealed samples were analyzed by electron diffraction experiments. It was confirmed that the initial samples contained various proportions of Iα (triclinic) and Iβ (monoclinic) phases. They could be classified into two families: a family I where the amount of the phase Iα was either small or below detection and a family II where this phase was the major component. The annealing treatment revealed that the Iα phase was metastable as it could be converted totally to the stable Iβ phase. The conversion Iα → Iβ was also followed by Fourier transform infrared (FT-IR) spectroscopy. Absorption bands near 3240 and 750 cm^{-1} were assigned to the Iα phase whereas bands near 3270 and 710 cm^{-1} corresponded to the Iβ phase.

Introduction

The recent discovery that cellulose I was a composite structure made of the two crystalline allomorphs Iα and Iβ is one of the major advances toward a full description of the crystalline structure of native cellulose. This result, which was first deduced by VanderHart and Atalla[1-3] from high-resolution CP/MAS ^{13}C NMR experiments, has now been confirmed by diffraction data.[4] In the emerging description of the two crystalline allomorphs of native cellulose, Iα is assigned to a triclinic form whose cell parameters are not yet firmly established, whereas Iβ is a two-chain monoclinic entity. In this scheme, all native celluloses may be classified into two families:[5] the family of *Valonia* and bacterial cellulose, where the cellulose is quite rich in the Iα phase, and the family of ramie and cotton, where it is Iβ that is dominant. In the Iβ family, tunicin is unique in the sense that this highly crystalline material consists uniquely of the Iβ allomorph.[6] One aspect of the Iα/Iβ dimorphism of cellulose is that Iα is metastable and can be converted readily and entirely into Iβ by hydrothermal treatment in the presence of NaOH.[7] In a similar fashion, the conversion Iα → Iβ can also be achieved by intracrystalline swelling followed by deswelling.[8] In this case, however, the resulting Iβ phase is of a much lower crystallinity than that obtained by the hydrothermal treatment.

Infrared spectroscopy is another solid-state technique that can be used to distinguish between cellulose samples from various origins. Marrinan and Mann were the first to point out that *Valonia* and bacterial cellulose gave infrared spectra that were different from those of tunicin, cotton, or ramie.[9,10] In particular, *Valonia* and bacterial cellulose presented an absorption band at 3242 cm^{-1}, which was absent in other celluloses. Marrinan and Mann could not explain this difference but suggested that it was "either due to a different crystal structure in the two cases or to a difference in degree of perfection of crystals having the same basic molecular arrangement". Liang and Marchessault[11] also observed the presence of a fairly intense band at 3245 cm^{-1} exclusively with *Valonia* and bacterial cellulose. According to these authors, the occurrence or not of this band had to be correlated more to specificities in orientation and degree of crystalline perfection than with different crystalline structures. Quite recently, Michell has applied the technique of second-derivative FT-IR to investigate a series of native cellulose samples.[12] The spectra that were obtained by this technique supported the hypothesis of Marrinan and Mann to classify the crystalline structures of native cellulose into two types: the algal–bacterial and cotton–ramie–wood types.

The possibility that now exists to obtain cellulose samples that are not only of high crystallinity but also of a definite Iα/Iβ composition should help in explaining the differences found in the infrared spectra of various cellulose materials. In particular, with samples that are rich in the Iα phase, the spectral difference resulting from hydrothermal annealing should enable one to attribute the absorption bands that are specific of this phase. This attribution is the aim of the present study where Fourier transform infrared (FT-IR) spectroscopy was applied to a series of highly crystalline cellulose samples from algal, bacterial, animal, or higher plant origins. In each case, infrared spectra were recorded before and after hydrothermal treatment and the Iα/Iβ conversion (if any) was followed by electron diffraction analysis. The present work demonstrates unambiguously that there are some infrared absorption bands that are specific of the Iα cellulose allomorph while others correspond to Iβ. As opposed to solid-state NMR spectroscopy or X-ray analysis, infrared and electron diffraction methods can be applied to very small amounts of material. These methods are therefore quite useful for measuring the local composition in Iα and Iβ phases when only small samples are available.

Experimental Section

Materials. *Valonia ventricosa* vesicles were harvested from the sea bed in the Lower Keys, FL. The cells were slit open and emptied and their walls were allowed to dry by pressing between two sheets of filter paper. The dried walls were boiled in 1% NaOH for 10 h and washed thoroughly before being immersed overnight in 0.05 N HCl at room temperature. As these purified walls sometimes gave an IR absorption band at 1600 cm^{-1} (attributed to hemicellulose contaminant), they were further purified by a treatment at 70 °C for 2 h in 0.3% $NaClO_2$, buffered at pH 4.9 in acetate buffer. This was followed by an overnight immersion in 5% KOH and a thorough washing in distilled water. The purified cell walls where the infrared band at 1600 cm^{-1} had either disappeared or strongly decreased were then stored in

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ethanol. Microcrystals of *Valonia* were prepared by refluxing purified *Valonia* cell wall with 2.5 N HCl for 8 h. The microcrystals were then washed thoroughly by centrifugation with distilled water. They were then stored in ethanol.

Bacterial cellulose from *Acetobacter xylinum* was cultivated for 12 days in Roux culture bottles. The resulting cellulose pads were washed under tap water for 2 days, extracted for 6 days with 0.5% NaOH, and washed with distilled water until neutrality was reached. The purified pads were then freeze-dried. Microcrystals of bacterial cellulose were prepared by refluxing pad fragments in 2.5 N HCl for 12 h. The microcrystals were washed extensively with distilled water and freeze-dried before storage.

Tunicates (*Salpa fusiformis*) were harvested in the Bay of Villefranche. The tunicates, preserved in ethanol, were slit open and most of the noncellulosic components were removed with tweezers. The cellulosic remainder (the tunicin) and the attached muscle were digested with pronase for 3 days at 40 °C. The tunicin was then extensively washed with distilled water and subjected to further pronase treatment for 48 h and subsequently washed. The sample was then refluxed with 1% NaOH under nitrogen for 4 h. After neutralization and washing, the sample was stored in ethanol until required.

Tunicin cellulose from the tests of *Halocynthia papillosa* preserved in ethanol was a gift from F. Gaill. Strips of the sample were pulled out with tweezers. They were boiled in 0.1 N NaOH for 6 h, rinsed, and boiled again in 0.2 N NaOH for another 6 h. After rinsing with distilled water, they were immersed in 0.1 N HCl for 6 h and washed thoroughly. These treatments were repeated twice after which the once colored sample became perfectly white. The samples were stored in 50% methanol/water. Microcrystals of *Halocynthia* were prepared by refluxing strips of purified tests with 2.5 N HCl. The microcrystals were then rinsed by successive centrifugation in distilled water and stored in ethanol.

Cellulose from *Rhizoclonium hieroglyphicum* was a gift from R. Atalla. The dried sample was extracted with hot methanol and acetone. It was then refluxed for 3 h in 1% NaOH, washed until neutrality, and subjected to a bleaching treatment for 2 h at 70 °C in 0.3% $NaClO_2$, buffered at pH 4.9 with acetate buffer. The sample was then washed thoroughly and freeze-dried. Microcrystals of *Rhizoclonium* were prepared by refluxing a purified sample in 2.5 N HCl for 3 h. The microcrystals were washed until neutrality and freeze-dried.

Bleached ramie cellulose was a gift from R. Hagège, at the French Textile Institute. Microcrystals of ramie were prepared by refluxing for 4 h in 2.5 N HCl. The microcrystals were then washed until neutrality and freeze-dried.

A sample of *Microdictyon tenuius* collected in the Bay of Villefranche was a gift from B. Caram. Its cell wall cellulose was purified by boiling it in 0.1 N NaOH for 10 h followed by rinsing in 0.05 N HCl under stirring and then distilled water until neutrality. The sample was then stored in methanol. Microcrystals of *Microdictyon* were prepared by boiling a purified specimen of this alga in 2.5 N HCl for 4 h. The resulting microcrystalline cellulose was then washed until neutrality and stored in distilled water to which a minute amount of NaN_3 was added as protectant.

A sample of purified cell wall from *Micrasterias denticulata* was a gift from W. Herth. Microcrystals of *Micrasterias* were prepared as those of *Microdictyon*.

A sample of *Glaucocystis nostochinearum* was a gift from R. Malcolm Brown, Jr. The sample was boiled with 0.1 N NaOH for 8 h. It was then neutralized for 12 h with 0.05 N HCl under stirring and then washed thoroughly in distilled water. Microcrystals of *Glaucocystis* were prepared as those of *Microdictyon*.

A sample of *Spirogyra* was a gift of R. Malcolm Brown, Jr. The sample was extracted with ethanol in a Soxhlet for 6 h. It was then boiled in 0.1 N NaOH for 4 h and rinsed in 0.05 N HCl overnight. The sample was still yellowish; it was bleached for 4 h at 70 °C in a 0.3% $NaClO_2$, buffered at pH 4.9 with an acetate buffer. Microcrystals of *Spirogyra* were prepared according to the method described for *Microdictyon*.

Hydrothermal Annealing. A small amount of each specimen was inserted in a small glass ampule that was filled with 0.1 N NaOH. The ampule was sealed and inserted in a 10-mL vapor pressure bomb to which a small amount of water was also added to build a counter pressure during the experiment. The bomb was hermetically sealed and heated in an oil bath to 260 °C. After it was kept at this temperature for 30 min, the bomb was cooled by quenching under tap water. The annealed specimen was then removed and washed thoroughly with distilled water until it reached neutrality. It was then dried for subsequent FT-IR analysis.

Electron Microscopy. All observations and diffraction experiments were achieved with a Philips EM 400 T electron microscope operated at 120 kV. Electron diffraction diagrams were recorded with electron doses kept to a minimum and the diagrams were recorded on Mitsubishi (MEM) emulsion.

Infrared Spectroscopy. Thin films of the specimens having a thickness between 5 and 20 μm were prepared. With unhydrolyzed *Valonia* and tunicin, small pieces of roughly 5 mm × 5 mm were cut from the samples. Thin layers were then delaminated under water and mounted with tweezers on microdisk sample holders perforated by a 500-μm hole. With cellulose microcrystals, a suspension in water was prepared by dispersion. A drop of the suspension was then allowed to dry in a small polyethylene cap. This gave thin films, which were mounted on microdisk sample holders.

A Fourier transform infrared (FT-IR) spectrometer (Perkin-Elmer 1720X), equipped with a microfocus accessory, was used. For polarized infrared spectra, the spectrometer was fitted with a gold wire grid polarizer. With polarized infrared, when a given band is greater with the radiation whose electric vector is perpendicular to the fiber axis than with the radiation parallel, the band is denoted as a perpendicular (⊥) band. When the reverse is true, the band is denoted as a parallel (∥) band. All the spectra were recorded in the transmission mode with a resolution of 2 cm^{-1}, in the range 4000–400 cm^{-1}.

Results and Discussion

Electron Diffraction Data. A study of the above samples by electron diffraction analysis indicates that their diffraction patterns can be catalogued into two families. Typical patterns of each family are shown in Figure 1. Figure 1A corresponds to a fiber diffraction diagram of an oriented part of a test of *Halocynthia*, whereas 1B is from a fragment of the cell wall of the alga *Microdictyon*.

In Figure 1A, each diffraction spot is symmetrically reflected in the four quadrants of the diagram. This diagram can be indexed totally according to a two-chains monoclinic unit cell with lattice parameters a = 0.800 nm, b = 0.817 nm, c (chain axis) = 1.036 nm, and γ = 97.2°. This diffractogram is identical with the one of annealed *Valonia* cellulose reported by Sugiyama et al.[4] From ^{13}C solid-state NMR studies, tunicin[6,7] and annealed *Valonia*[13] have been described as being of a pure Iβ phase. Thus, the pattern in Figure IA confirms that the Iβ phase of cellulose corresponds indeed to a monoclinic crystal structure with two cellulose chains in the unit cell.

The fiber diagram of *Microdictyon* cellulose (Figure 1B) is of high orientation and resolution. It displays not only the reflections observed in the diagram of *Halocynthia* but also extra spots that can be observed in every layer line with the exception of the equator. In particular, four of these additional reflections (marked with arrows in Figure 1B) are well separated from the monoclinic reflections. These extra spots are not symmetrically reflected in the four quadrants of the diagram but are only present as Friedel pairs. Therefore, these spots indicate the presence of a triclinic phase. The diagram in Figure 1B contains all the reflections of the initial *Valonia* diagram reported by Honjo and Watanabe.[14] In order to index it completely, one requires either a large eight-chain triclinic unit cell or a two-phase system containing at least one triclinic component. Following the reasoning of Sugiyama et al.,[4] the two-phase system is favored. Thus, the electron diffractogram of *Microd-*

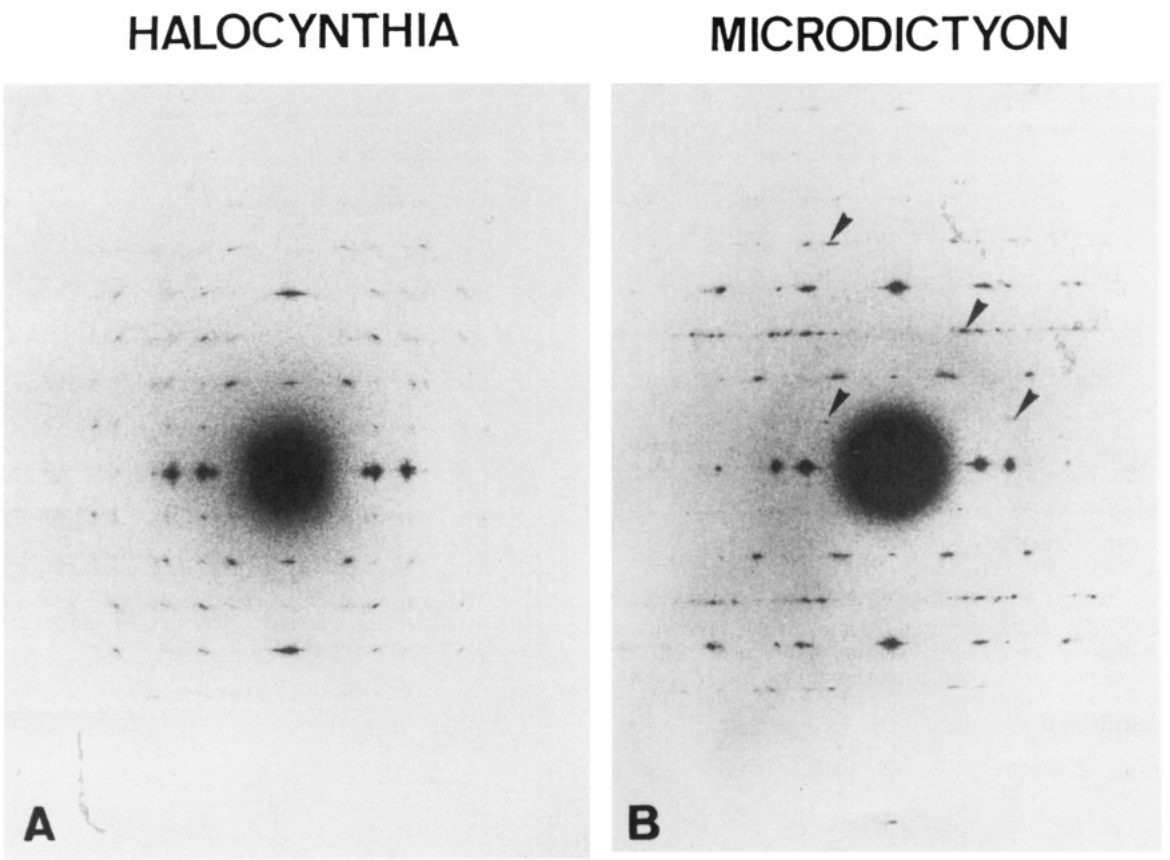

Figure 1. Fiber electron diffraction diagrams taken from a bundle of cellulose microfibrils from *Halocynthia* (A) and *Microdictyon* (B). The arrows in part B correspond to reflections that can be assigned unambiguously to the triclinic phase Iα.

Table I
The Two Families of Native Cellulose[a]

family I, samples from	family II, samples from
Salpa tunicin	initial *Valonia*
Halocynthia tunicin	initial *Microdictyon*
Micrasterias	initial *Glaucocystis*
Spirogyra	initial *Rhizoclonium*
ramie	initial bacterial cellulose from *Acetobacter*
annealed *Valonia*	
annealed *Microdictyon*	
annealed *Glaucocystis*	
annealed *Rhizoclonium*	
annealed bacterial cellulose from *Acetobacter*	

[a] A study of the electron diffractograms indicates that in family I only the monoclinic Iβ phase is present. In family II there are two phases: the monoclinic Iβ and the triclinic Iα.

ictyon contains the same information as that of *Valonia*: it can be considered as being the superposition of the monoclinic diagram shown in Figure 1A to that of a triclinic cellulose structure having two chains per unit cell,[4] similar to that proposed by Sarko and Muggli.[15] The two diagrams have many overlapping diffraction spots. This is particularly true for the equator and the second and fourth layer lines, which are almost identical in both of them. Thus, the two corresponding crystal structures must have features that are closely related. Among these, their *ab* projections must be similar.

In Table I, the samples are listed into two families according to the two types of diffraction diagrams. In family I, ramie, *Micrasterias*, *Spirogyra*, and *Salpa* tunicin as well as all the annealed samples present diffraction diagrams that display the same features as those of *Halocynthia* shown in Figure 1A. These samples contain essentially the Iβ phase. On the other hand, in family II, the unannealed samples of *Valonia*, *Rhizoclonium*, bacteria, and *Glaucocystis* gave diffraction diagrams that have the same two-component features as that of *Microdictyon* in Figure 1B. Thus, they also consist of a mixture of Iα and Iβ.

Infrared Spectroscopy. When the samples were analyzed with infrared spectroscopy, most of them gave well-resolved spectra. The annealing treatment brought minor modifications to the spectra of some of the specimens especially in the regions 3500–3200 and 1000–400 cm^{-1} but did not affect their resolution, which seemed as high before as after annealing. As for the acid hydrolysis, it did not bring any significant change in the infrared spectra nor in their resolution. For this reason, thin films made from acid-prepared microcrystals were routinely made and used throughout this study.

Following the classification of Table I, it was found that the samples listed in family II displayed two absorption bands that were not present in family I. This is illustrated in Figures 2–6 and presented in Tables II and III. In Figure 2, two spectra of *Rhizoclonium* cellulose are shown, corresponding to the initial unannealed and to the annealed sample. In the initial sample, a band near 3240 cm^{-1} and one near 750 cm^{-1} are clearly visible: these two bands disappear upon annealing. On the other hand, two other bands, namely, those near 3270 and 710 cm^{-1}, become stronger as a result of the annealing treatment.

In Figure 3 and 4, which are enlargements of the 4000–2400- and the 1000–400-cm^{-1} regions of the spectra, the effects of the annealing treatment are compared between *Rhizoclonium* and *Halocynthia* cellulose. Unlike *Rhizoclonium* cellulose, *Halocynthia* does not show any band at 3240 cm^{-1} or at 750 cm^{-1}. Furthermore, its spectrum appears totally unaffected by the annealing treatment. As for the bands at 3270 and 710 cm^{-1}, they are somewhat stronger in both *Halocynthia* spectra than in that of the

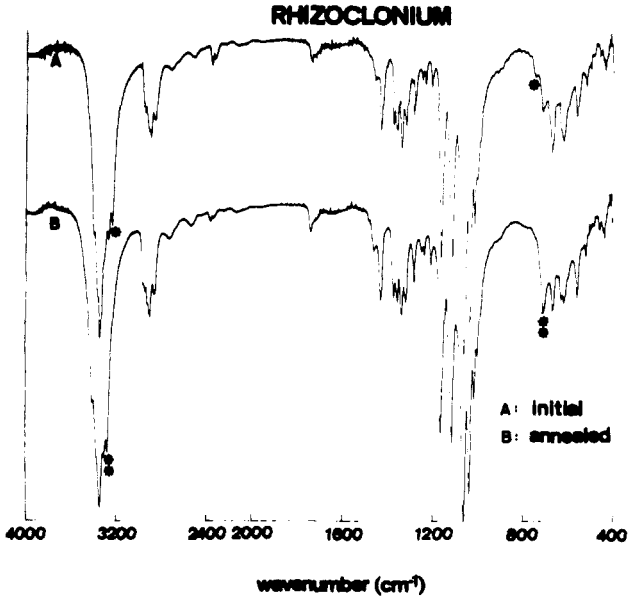

Figure 2. FT-IR spectra of initial (A) and annealed (B) *Rhizoclonium* cellulose. In the initial sample, bands near 3240 and 750 cm^{-1} corresponding to the Iα phase (identified by *) disappear during annealing. Bands near 3270 and 710 cm^{-1} (identified by **) are specific of the Iβ phase.

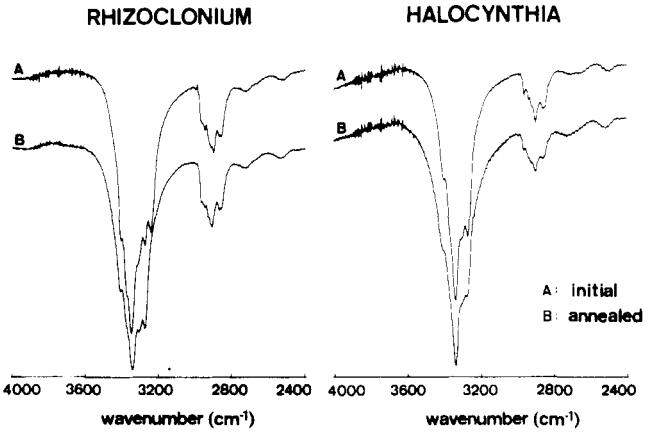

Figure 3. Details of the FT-IR spectra in the region 4000–2400 cm^{-1} of cellulose from *Rhizoclonium* and *Halocynthia* before (A) and after annealing (B).

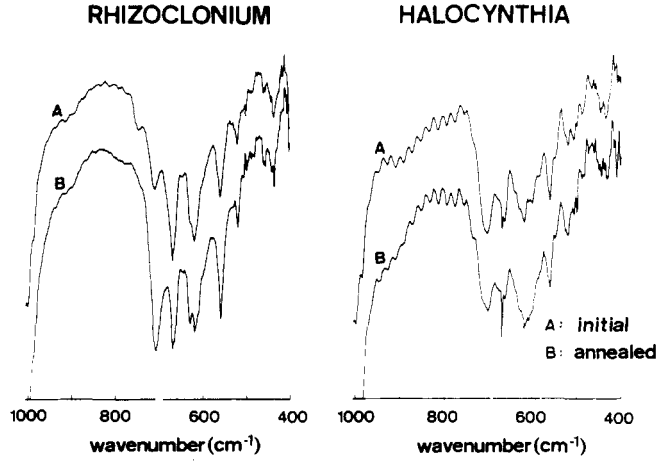

Figure 4. Details of the FT-IR spectra in the region 1000–400 cm^{-1} of cellulose from *Rhizoclonium* and *Halocynthia* before (A) and after (B) annealing.

initial *Rhizoclonium*. On the other hand, these two bands are comparable in strength both in the *Halocynthia* and in the annealed *Rhizoclonium* specimen.

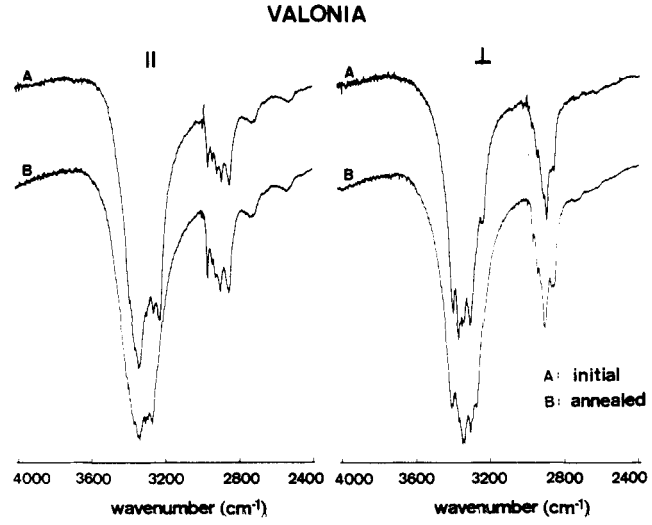

Figure 5. Details of the FT-IR with polarized radiations in the region 4000–2400 cm^{-1} of cellulose from *Valonia* before (A) and after (B) annealing. The spectra obtained with parallel (∥) radiations are on the left. Those with perpendicular (⊥) radiations are on the right.

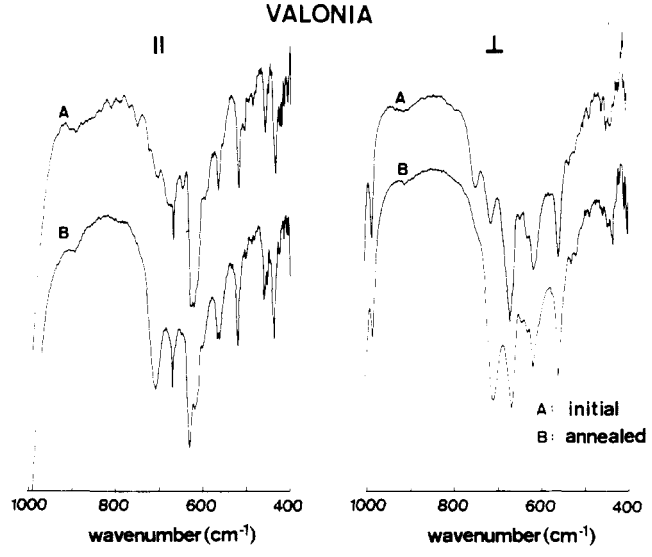

Figure 6. Details of the FT-IR spectra with polarized radiation in the region 1000–400 cm^{-1} of cellulose from *Valonia* before (A) and after (B) annealing. The spectra obtained with parallel (∥) radiation are on the left. Those with perpendicular (⊥) radiations are on the right.

Table II
Infrared Bands for Cellulose in the OH Stretching Region (3500–3200 cm^{-1})

frequency, cm^{-1}		
family I[a]	family II[b]	polarization
3409	3401	⊥
3365	3369–70	⊥ questionable[c]
3339–50	3339–50	∥
3306	3306	⊥
3270	3270	questionable[c]
none	3237–42	∥

[a] Cellulose from Tunicates (*Salpa* and *Halocynthia*), *Micrasterias*, *Spirogyra*, ramie, and annealed samples. [b] Cellulose from initial *Valonia*, *Rhizoclonium*, *Microdictyon*, *Glaucocystis*, and *Acetobacter*. [c] In the annealed samples, the polarization of this band is not obvious.

In Figures 5 and 6 are represented the polarized spectra of initial and annealed *Valonia* cellulose, which belongs initially to family II and get converted to family I after annealing. These polarized spectra show that the band

Table III
Infrared Bands for Cellulose in the Region 1000–400 cm^{-1}

frequency, cm^{-1}		
family I[a]	family II[b]	polarization
none	750	⊥
706–09	711–12	⊥ questionable[c]
667–68	667–68	composite band[d]
617–18	617–18	composite band[d]
558–62	558–62	composite band[d]
519–20	519–20	∥

[a] Cellulose from Tunicates (*Salpa* and *Halocynthia*), *Micrasterias*, *Spirogyra*, ramie, and annealed samples. [b] Cellulose from initial *Valonia*, *Rhizoclonium*, *Microdictyon*, *Glaucocystis*, and *Acetobacter*. [c] The polarization of this band, which seems perpendicular in the initial specimens, appears questionable in the annealed samples. [d] They are composed of several bands. Their polarization is not obvious.

near 3240 cm^{-1} has a parallel polarization, whereas for that at 710 cm^{-1}, the polarization is perpendicular. The bands at 3270 and 750 cm^{-1} appear to be unpolarized.

The list of the main infrared bands in the regions 3500–3200 and 1000–400 cm^{-1} together with their polarization behavior are listed in Tables II and III.

The infrared bands in the region 3600–3200 cm^{-1} correspond to the OH stretching frequencies of cellulose. The polarization behavior as well as the precise interpretation of some of these bands has been discussed by Liang and Marchessault.[11] These authors have indicated that several of the OH stretching bands corresponded to specific intramolecular hydrogen bonds of cellulose. In particular, they could assign the perpendicular bands at 3305 and 3405 cm^{-1} to intermolecular hydrogen bonds along the (1$\bar{1}$0) and (110) planes. (These indices refer to the cellulose unit cell defined by Gardner and Blackwell.[16]) For the parallel band at 3350 cm^{-1}, Liang and Marchessault tentatively interpreted it as corresponding to an intramolecular hydrogen bond. In the case of the OH stretching bands, which are of interest here, the polarization is parallel for the band near 3240 cm^{-1} but unpolarized for that near 3270 cm^{-1}. So far, the assignment of these two OH stretching bands could not be established with certainty.

One of the interesting features presented here as a result of the annealing experiment is that the band near 3240 cm^{-1} can be assigned to the triclinic Iα cellulose whereas that near 3270 cm^{-1} seems to be proportional to the amount of monoclinic phase Iβ; therefore, it can be assigned to the monoclinic Iβ cellulose. In agreement with this assignment, the annealing treatment, which converts cellulose Iα into Iβ, eliminates completely the band near 3240 cm^{-1}, which is polarized in a parallel mode. During the conversion, the new band near 3270 cm^{-1} has lost its polarization. This indicates that in the process, some of the hydrogen bonds that were parallel to the cellulose chain axis are reorganized in a different way when the solid-state transformation I$\alpha \rightarrow$ Iβ takes place. The fact that only the OH stretching frequencies are affected by the transformation seems to imply essentially a reorganization of the hydrogen bonds during the I$\alpha \rightarrow$ Iβ transformation. Our observations are thus in full agreement with the Raman analysis of Wiley and Atalla.[17] These authors were able to propose, as we do, that Iα and Iβ celluloses had closely related molecular conformations but different hydrogen bonding patterns.

In the region 800–600 cm^{-1}, the infrared spectra of cellulose are more difficult to interpret. Furthermore, due to many overlaps, the polarization of the various bands in this region is not always straightforward. Nevertheless the band near 750 cm^{-1}, which appears only in the spectra of family II, has a marked perpendicular polarization. This band disappears during the annealing treatment and seems to be replaced by the unpolarized band near 710 cm^{-1} which is markedly increased in all the samples listed in family I. Thus these two bands seem to correspond respectively to the Iα and Iβ phases of cellulose. Blackwell et al.[18] have proposed that the band near 750 cm^{-1} in *Valonia* corresponded to a CH_2 rocking mode of cellulose. The fact that no Raman absorption band exists in this region[17,18] raises a question concerning this assignment, which we prefer to leave open.

An interesting aspect of the present study is that it clarifies the discrepencies that were found between the infrared spectra of highly crystalline native cellulose samples such as tunicin or *Valonia*.[10] The present FT-IR results are in good agreement with the ^{13}C solid-state NMR[1-3,5] and the crystallographic data,[4] which indicated not only that all native celluloses consisted of a mixture of two crystalline phases, Iα and Iβ,[2] but also that they could be classified into two families.[5] One advantage of the FT-IR technique over that of ^{13}C solid-state NMR is that it requires only a very small amount of sample and therefore is quite appropriate for the identification of small specimens.

The question that remains to be answered is the origin of the biphasic character of native cellulose. Sugiyama et al.[4] have proposed that the Iα phase was due to built-in strains occurring during cellulose biosynthesis. It is interesting to notice that, at least for three of the samples in family II (*Valonia*, *Microdictyon*, and *Glaucocystis*) that are rich in Iα, the cellulose is biosynthesized by fairly large linear terminal complexes (TCs).[19] At least for *Valonia*, these linear TCs produce relatively large cellulose microfibrils having nearly square sections.[20,21] On the other hand, for *Micrasterias* and *Spirogyra* cellulose, which are of family I, the TCs are made of rosettes organized in array.[19] At least in the case of *Micrasterias*, these TCs produce ribbon like microfibrils of very narrow thickness.[22] Even if these two types of TCs synthesize cellulose molecules along the same biochemical mode, their morphological difference may strongly influence the crystallization of the nascent cellulose microfibrils. In particular, with the large linear TCs, the structure of the emerging cellulose microfibril may be strongly influenced by the organization of the TCs themselves. This interaction may be responsible for the buildup of strains within the nascent microfibrils, thus leading to the occurrence of the metastable Iα phase. Such a situation may be quite different from what occurs when the microfibrils are generated by the rosettes. These rosettes, being much smaller than the linear TCs, could very well yield strain-free cellulose microfibrils crystallized entirely in the stable monoclinic Iβ phase. In the light of this hypothesis, the highly crystalline Iβ *Halocynthia* cellulose appears to be the ideal strain-free Iβ sample. It would be of much importance to investigate its mode of biosynthesis and in particular to visualize its TCs in order to see whether they are of the linear or the rosette type.

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