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SOLID-STATE ^{13}C-N.M.R. AND ELECTRON MICROSCOPY STUDY ON THE REVERSIBLE CELLULOSE I→CELLULOSE III_I TRANSFORMATION IN *Valonia*

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ABSTRACT

An electron microscopy and a high-resolution ^{13}C-n.m.r. study of cellulose is described and a previously unreported solid-state ^{13}C-n.m.r. spectrum of cellulose III is presented. It is shown that a cyclic transformation of highly crystalline cellulose I (*Valonia*) into cellulose III and back to cellulose I results in a material whose crystallites have a reduced lateral dimension similar to that in cotton and whose electron-diffraction patterns and ^{13}C-n.m.r. spectra are very close to that of cotton. In addition, some new features in the ^{13}C-n.m.r. spectrum of *Valonia* cellulose are reported.

INTRODUCTION

High-resolution solid-state n.m.r. spectroscopy has been widely used for structural studies on cellulose[1–7]. From the four different polymorphic forms of cellulose which have been identified, it is found that high-resolution solid-state ^{13}C-n.m.r. can, on the basis of differing chemical shifts, readily distinguish between cellulose I, II, and IV (ref. 8).

Native cellulose (cellulose I) is often heterogeneous, containing highly ordered or crystalline components and material existing in regions of packing disorder. The disordered regions, and also it is believed material on the surface of crystallites, produce rather broad peaks or shoulders in the n.m.r. spectrum. The carbon nuclei in more-ordered regions are associated with relatively well-resolved

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peaks where, in general, the cross-polarisation signal intensity is proportional to the number of carbon nuclei giving rise to that signal[9].

The appearance of the narrower peaks in the cellulose I spectrum is governed by the origins of the sample (for example, *Valonia* cellulose, bacterial cellulose, cotton, etc.). Multiplicities have been observed (particularly in the resonances arising from C-1, C-4, and C-6), the relative intensities of the components of these multiplets being sample-dependent. Thus *Valonia* cellulose has C-1 and C-4 resonances which are each three-component multiplets, with the central component being the most intense. Two different interpretations have been proposed to account for the variations in the spectra of native cellulose. One hypothesis[10] proposes that the more-ordered component of native cellulose is composed of two forms, I_α and I_β, and thus any cellulose I sample is considered to be made up of varying amounts of these two forms. For *Valonia* cellulose, the three-component multiplet for C-1 in the ^{13}C-n.m.r. spectrum would arise from a linear combination of an intense single line (attributed to the I_α form) surrounded by two weaker resonances assigned to I_β. The alternative explanation[11] of cellulose I spectra is based on a comparison of n.m.r. with X-ray diffraction data and also proposes the existence of two forms of native cellulose. It is suggested that the cellulose I sample is made up of varying amounts of two types of material that are characterised either by an eight-chain unit cell or a two-chain unit cell. Thus peaks due to C-1 and C-4 in the ^{13}C-n.m.r spectrum of a given sample of native cellulose would arise from a linear combination of a 1:1·2 multiplet due to the eight-chain unit-cell material and a 1:1 multiplet due to the two-chain material. Thus *Valonia* cellulose, it is argued, with 1:1:2 multiplets for C-1 and C-4, is made up entirely of material that is characterised by an eight-chain unit cell

The present work is an attempt to throw more light onto the interpretation of cellulose I spectra. To this end, a highly crystalline material, *Valonia* cellulose microcrystals, was converted into a sample of lower crystallinity. The conversion was achieved by a series of cyclic solid-state treatments where *Valonia* cellulose was transformed into cellulose III_I, and then converted back into cellulose I. Such changes were shown by electron microscopy to partially decrystallise cellulose by reducing the lateral width of the initial crystals[12]. The transformations were studied using the techniques of solid-state ^{13}C-n.m.r., electron microscopy, and electron-diffraction analysis. In addition to the study of the various cellulose I samples, the intermediate cellulose III_I was also investigated, as, thus far, no spectrum has been reported in the literature for this polymorph

EXPERIMENTAL

Cellulose samples. — *Valonia ventricosa* cellulose, collected in Florida, was purified as described previously[13]. Microcrystals of *Valonia* cellulose (cellulose I) were prepared according to the method of Chanzy and Henrissat[14]. Transformation into cellulose III_I was achieved by successive treatments in anhydrous

ethylenediamine alternating with washings in anhydrous mehanol[15]. The conversion of cellulose III$_I$ back into cellulose I was carried out by heating a water suspension of the sample in a manner already described[12], except that here the treatment temperature was 160°.

High-resolution ^{13}C-n.m.r. spectroscopy. — High resolution solid-state ^{13}C-n.m.r. spectra were obtained with a Bruker CXP 300 instrument operating at 75.46 MHz for ^{13}C. Cross-polarisation–magic-angle spinning techniques (c.p.–m.a.s) were employed with single contact pulses of 1–5 ms duration and recovery times between acquisitions of at least 5 s. Spectra were acquired using a modest spinning rate of 3 kHz, which was sufficient to suppress problems associated with spinning sidebands. A proton decoupling strength of ~50 kHz was applied at approximately the frequency of the methylene-group protons. The ^{13}C chemical shifts are quoted with respect to tetramethylsilane and were obtained relative to the shifts of adamantane (assumed to be 29.5 and 38.6 p.p.m. from Me_4Si). The ^{13}C chemical-shift values quoted for all of the peaks in the cellulose I spectra are ~0.8 p.p.m. lower than those quoted elsewhere[8,9]. Resolution enhancement for data shown in Fig. 2 was performed by multiplication of the free-induction decay using a function of the form: $\exp\{-at - bt^2\}$, where $a = \pi \times$ LB and $b = -a/2 \times$ GB $\times$ AQ. The line broadening LB = −60 Hz, the Gaussian broadening factor GB = 0.5, and the acquisition time AQ = 26 ms.

Electron microscopy. — A Philips EM 400T microscope was used throughout. Images were recorded at a plate magnification of 28,000 and at an accelerating voltage of 80 kV on specimens negatively stained with uranyl acetate. Electron-diffraction diagrams were recorded on unstained specimens at 120 kV under conditions of minimum exposure.

RESULTS

Valonia cellulose occurs in the algal cell-wall as almost endless microfibrils having a ~20 × 20 nm square cross-section[16]. Upon treatment with hydrogen chloride in anhydrous methanol, these microfibrils are transformed into micro-crystals having the same lateral dimensions and crystalline perfection, but whose length is considerably decreased and ranges from 500 nm to several μm (Fig. 1)[14].

High-resolution ^{13}C-n.m.r. spectra of the initial *Valonia* cellulose and of the microcrystals are shown in Fig. 2A and 2B, respectively. These spectra were acquired using a higher field than in previous studies and have been resolution-enhanced. They are similar both to each other and to spectra published previously[3,9]. The modest resolution-enhancement results in the splitting of the C-1 and C-4 resonances almost to the baseline and reveals a number of features in the spectra. There are two high-frequency shoulders in the resonance at ~71 p.p.m. and the signal at ~66 p.p.m. (assigned to C-6) is broad. The multiplet at ~90 p.p.m., assigned to C-4, consists of a relatively narrow and intense central component surrounded to high and low frequency by somewhat broader and less-

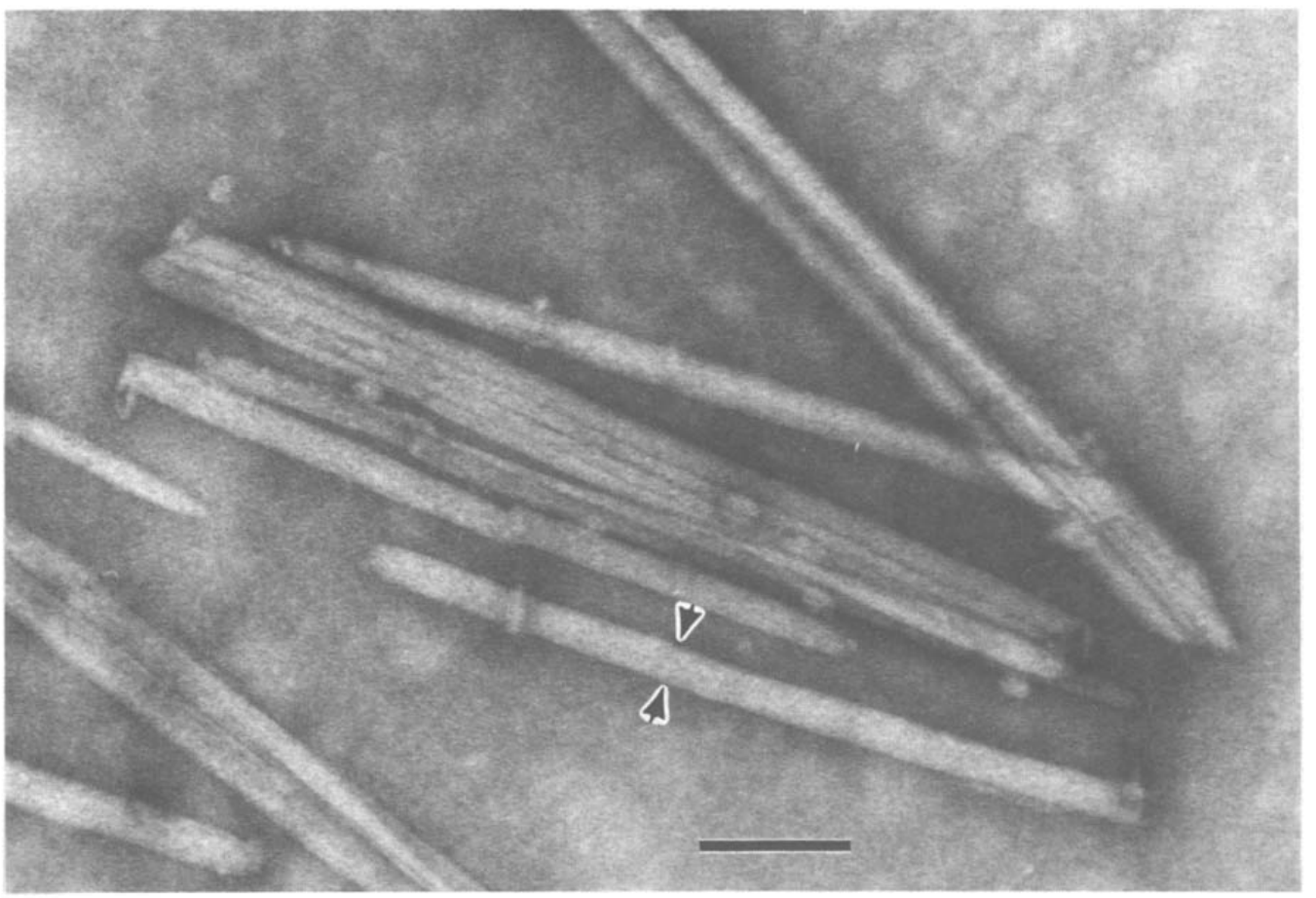

Fig 1 Electron micrograph of *Valonia* cellulose microcrystals after negative staining with uranyl acetate A typical microcrystal such as the one between arrows has a width of 20 nm. Scale bar = 100 nm

intense peaks. The smallest of the three peaks (at 88.6 p.p.m.) shows a slight asymmetry. These features are not a consequence either of poor shimming or digitisation, or of the resolution-enhancement process in that they appear reproducibly in a number of spectra of *Valonia* cellulose that we have acquired. After treatment with ethylenediamine, and washing in methanol, *Valonia* cellulose microcrystals (polymorph I) are transformed in the polymorph III_I. At the ultra-structural level, this conversion induces a fibrillation of the initial microcrystals into sub-elements displaying an unchanged length but a width decreased to 3–5 nm (Fig. 3). Electron diffraction, recorded on a bundle of oriented microcrystals, clearly shows that the transformation of cellulose I into cellulose III_I is complete (Fig. 4). The electron-diffraction diagram of oriented *Valonia* cellulose microcrystals, polymorph I (Fig. 4A), is well resolved and the resolution on the meridian goes beyond 0.10 nm^{-1}, whereas it reaches 0.20 nm^{-1} on the equator. Several hundred diffraction maxima are observed and, in particular, sharp equatorial maxima at d = 0.60, 0.54, and 0.39 nm, corresponding to the $(1\bar{1}0)$, (110), and (020) planes respectively[13]. On the meridian, the dominating intensity is attributed to the (004) planes at d = 0.258 nm. In addition, it is noted that the relative intensities of the (020) and (110) reflections are approximately equivalent in the electron-diffraction diagram of *Valonia* cellulose microcrystals (Fig. 4A), whereas the intensity of the

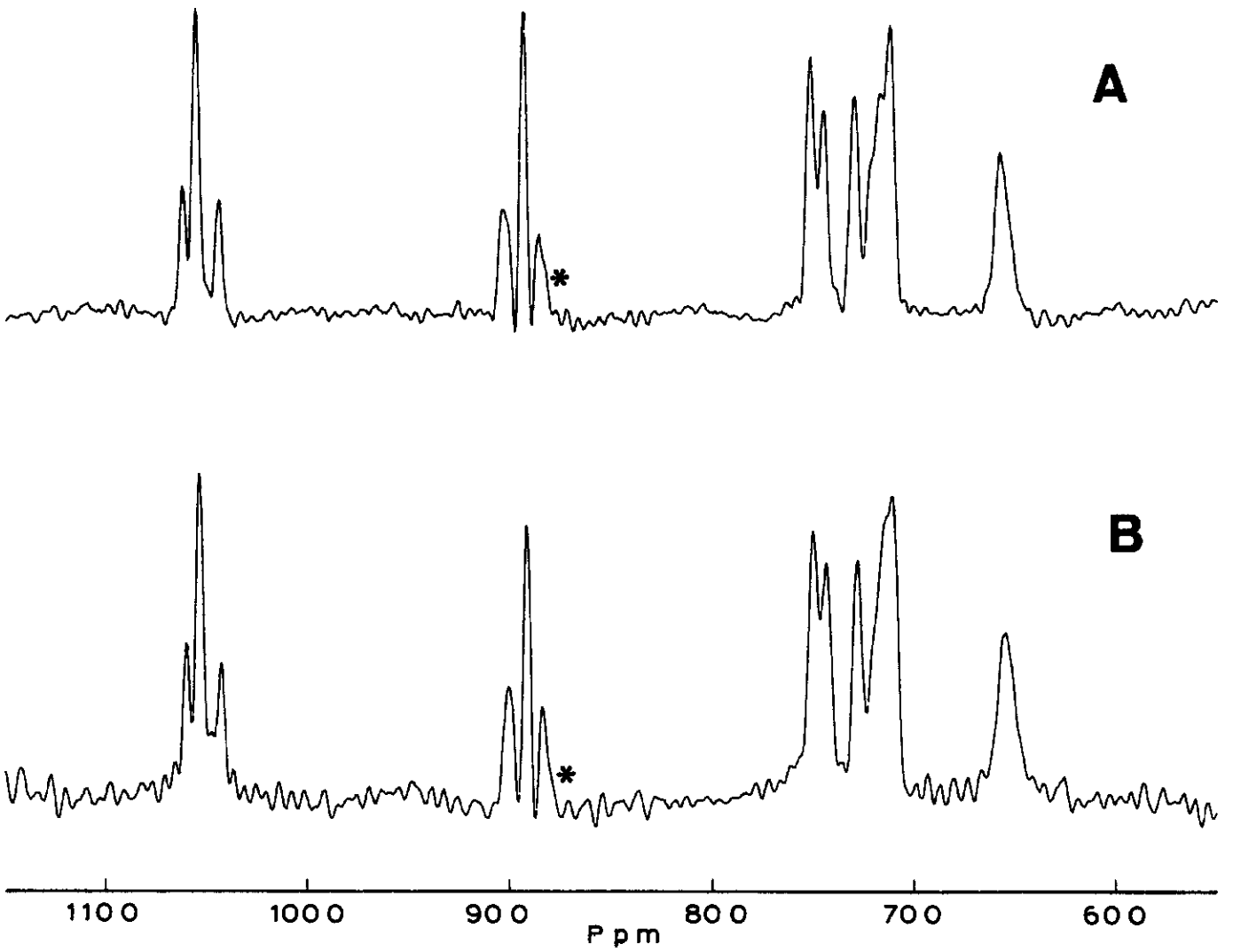

Fig 2 Resolution-enhanced 75-MHz ^{13}C solid-state n m r spectra of. *A*, initial *Valonia* cell-wall fragments, *B*, *Valonia* cellulose microcrystals The asterisks indicate the presence of a shoulder on the peak (see text)

($1\bar{1}0$) reflection is drastically reduced. This results from a preferential orientation of the crystallites with their ($1\bar{1}0$) face on the supporting carbon used for the electron-microscopy preparation.

The electron-diffraction pattern recorded from oriented cellulose polymorph III$_I$ (Fig. 4B), is typical of the cellulose III$_I$ lattice, with the ($1\bar{1}0$), (110), and (020) reflections located on the equator at d = 0.750, 0.420, and 0.420 nm, respectively[17]. Among meridional reflections, the dominating intensity is now at d = 0.516 nm and corresponds to (002) planes, whereas (004) planes remain unchanged at d = 0.258 nm.

The ^{13}C-n.m.r. spectrum of cellulose III$_I$ (Fig. 5) shows only six clearly resolved peaks, although the C-1, C-4, and C-6 resonances are broadened at the base, suggesting that amorphous and/or surface material is contributing to these signals. The transformation of cellulose I into cellulose III$_I$ is accompanied by a dramatic change in spectral appearance. The largest change in chemical shift (3.1 p.p.m.) occurs at C-6. Chemical-shift values of cellulose I and III$_I$ are shown in Table I.

Changing cellulose III$_I$ back into cellulose I$_{III}$ is achieved by a high-temperature water treatment[12]. Electron microscopy, in the image mode, shows no notice-

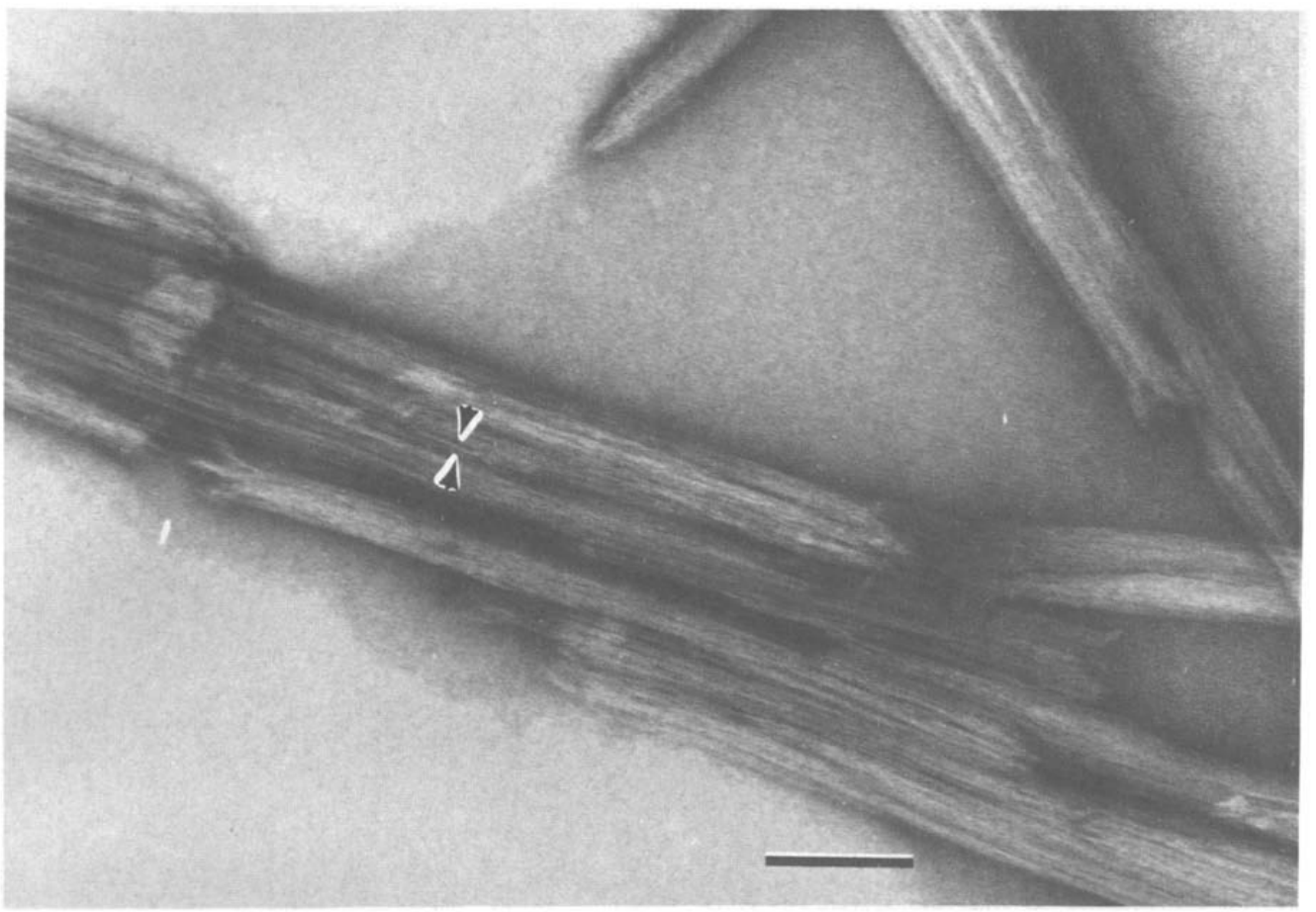

Fig 3 Identical to Fig 1 but after conversion into cellulose III_I Individual crystals have now a width of ~3–5 nm Scale bar = 100 nm

able morphological changes with respect to cellulose III_I. However, electron diffraction of such oriented microcrystals shows that this conversion is complete (Fig. 4C).

The new diffraction diagram is typical of a cellulose I lattice with, however, a definition much poorer than that of the starting *Valonia* cellulose. As such, the

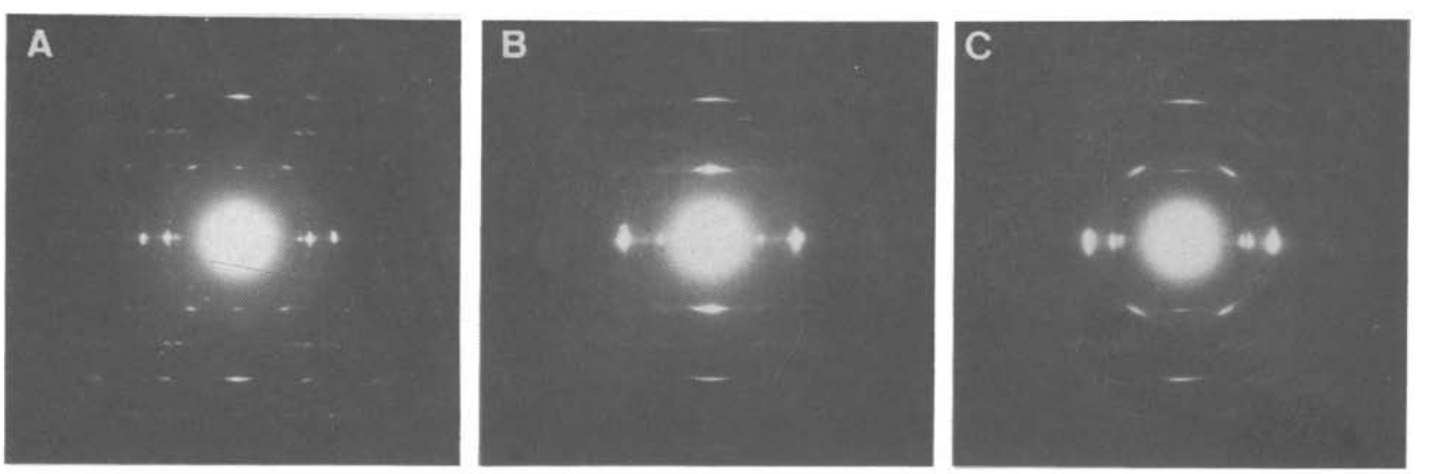

Fig 4 Electron-diffraction diagram of a bundle of microcrystals with vertical axis of *A*, initial *Valonia* cellulose microcrystals, *B*, identical to *A* but after conversion into cellulose III_I, and *C*, identical to *B* but after conversion back into cellulose I (cellulose I_{III})

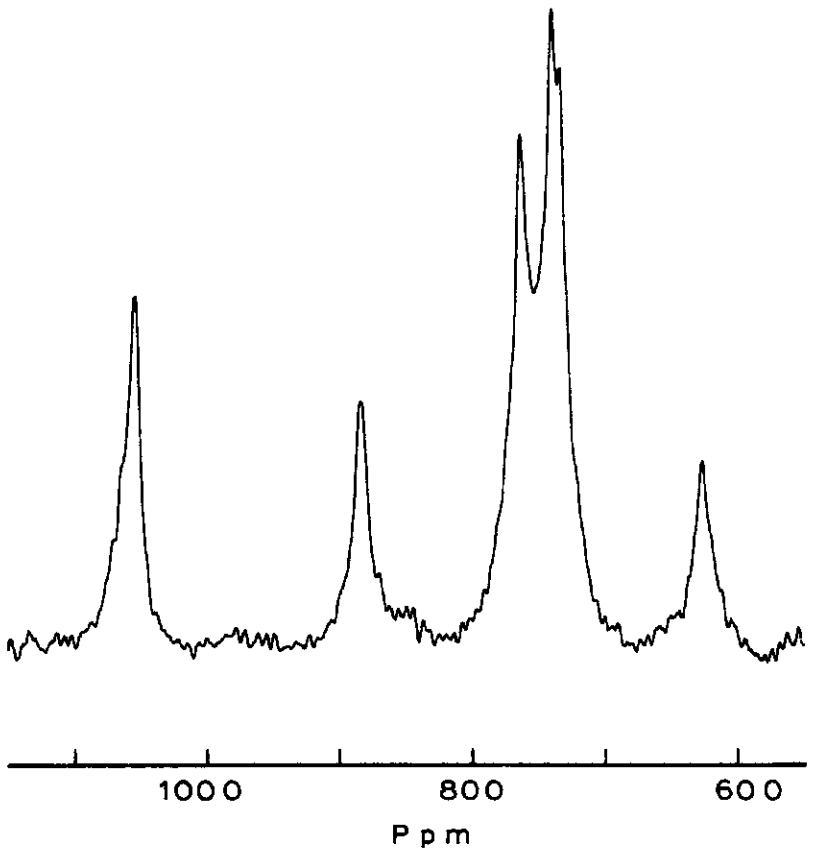

Fig 5 The ^{13}C solid-state n.m r spectrum of cellulose III$_I$ at 75 MHz

diffraction pattern in Fig. 4C bears a close resemblance to the diffraction diagrams recorded with ramie or cotton. The broader width of the diffraction spots in the I_{III} sample indicates a decreased lateral size of the crystallites[12]. In addition, in the cellulose I_{III} diffraction diagram, the (020) reflection is now the strongest among the equatorial intensities. This contrasts with the starting *Valonia* cellulose microcrystals, and indicates that the cellulose crystallites no longer display preferential orientation on the carbon surface of the specimen holder. This is further confirmed by the intensity of the $(1\bar{1}0)$ reflection, which now approaches that of (110).

The ^{13}C-n.m.r. spectrum of cellulose I_{III} (Fig. 6A) is typical of cellulose I and,

TABLE I

^{13}C CHEMICAL SHIFTS (δ, P P M) OF CELLULOSE SAMPLES

Sample	*Atom*			
	C-1	*C-4*	*C-2, C-3, C-5*	*C-6*
Valonia (cellulose I)[a]	106 0 105 5 104 4	90.2 89.3 88.6	75 1 74 5 72 9 72 1 71 7 71 3	65 7
Cellulose III$_I$	105 3	88.3	76.3 73.8 73 3	62 6

[a]These values are 0 8 p p m lower than those quoted in other studies[8,9]

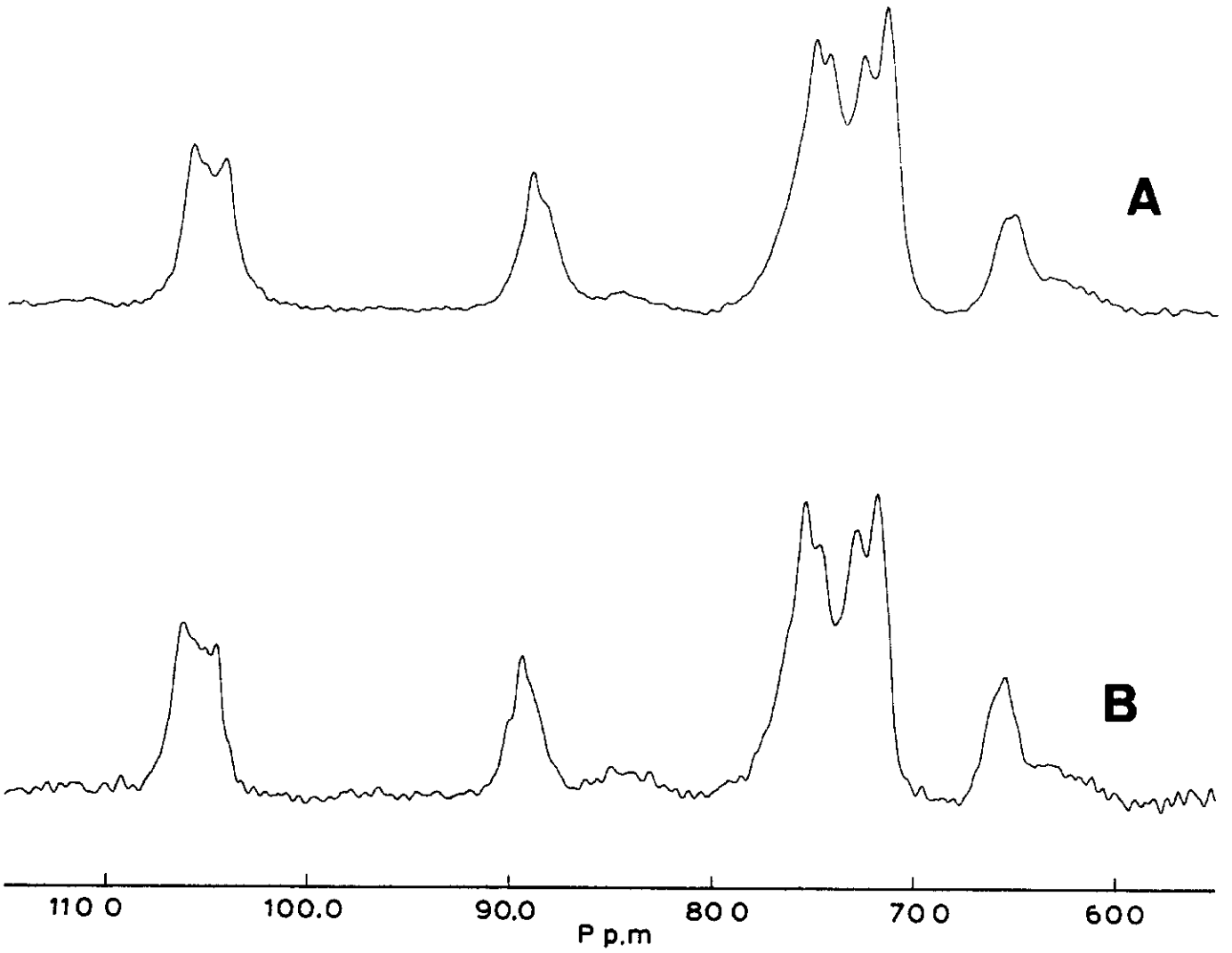

Fig 6 The ^{13}C solid-state n.m r spectra at 75 MHz of· *A*, *Valonia* cellulose I_{III}, *B*, cotton (cellulose I)

in particular, the chemical shift of C-6 has returned to its original position at 65.7 p.p.m. In addition, this spectrum shows a striking resemblance to that recorded from cotton cellulose (Fig. 6B), with similar peak-intensities and multiplicities and a similar proportion of amorphous domains.

DISCUSSION

In the present study we have combined three complementary techniques, namely solid-state ^{13}C-n.m.r., electron diffraction, and electron microscopy to obtain information on various solid-state treatments applied to native cellulose.

The initial acid treatment, which was applied to *Valonia* cellulose in order to prepare the microcrystals, has no effect either on its ^{13}C-n.m.r. spectrum—in particular the three-component multiplets of C-1 and C-4—or its electron-diffraction pattern. On the other hand, the longitudinal sizes of the cellulose crystals are drastically reduced from infinite *Valonia* microfibrils to micrometer-sized crystals (Fig. 1). Thus an important parameter, for both n.m.r. and diffraction data, appears to be the lateral width of the crystals, which is related to their crystalline perfection.

When the *Valonia* cellulose microcrystals undergo a transition to cellulose

III_I followed by another transition back to "native" cellulose I_{III}, a large number of longitudinal defects are created as the crystals become sub-fibrillated into sub-elements 3–5 nm wide (Fig. 3). This has an effect both on the diffraction diagram, which loses its equatorial resolution, and on the ^{13}C-n.m.r. spectrum. Such modifications are well illustrated when comparing the shape of the signal attributed to C-1 in the initial spectrum (Fig. 2B) and the final spectrum (Fig. 6A). Quite remarkably, cellulose I_{III}, which is now made of sub-elements having a lateral width comparable to those found in cotton—namely 3–5 nm—, presents a ^{13}C-n.m.r. spectrum almost identical to that of cotton. This is seen when comparing Figs. 6A and 6B, especially in the C-1 region. It is interesting to consider the origins of these similarities. Two possible explanations are evident. The solid-state transformation may have generated, quite by chance, a material (I_{III}) identical to cotton in that it contains not only similar amounts of surface/disordered components but also identical amounts of the I_α and I_β forms (or 2-chain and 8-chain unit-cell material) as cotton. A more likely interpretation is that it is the lateral width of the crystals which determines the appearance of the ^{13}C-n.m.r. spectrum of native cellulose.

One of our initial goals was to bring some evidence to favour either the hypothesis of Atalla and VanderHart[10] or that of Cael *et al.*[11] in the interpretation of the fine details of the solid-state ^{13}C-n.m.r. spectra of cellulose. With the results presented here, it is not possible to substantiate either theory. Indeed, if the initial *Valonia* consisted of a mixture of two phases, I_α and I_β, our experiments demonstrate that during the transformation of *Valonia* cellulose into cellulose III_I and then back to cellulose I, most of the I_α phase is converted into I_β, obviously of lower perfection. On the other hand, if the hypothesis of 8- and 2-chain unit cells is the correct one, the transformation converts most of the 8-chain unit-cell material into the 2-chain type.

The interpretation of multiplicities in c.p.–m.a.s. ^{13}C-n.m.r. spectra can be a problem. It has been shown[18] that two relatively simple, isomorphous crown-ether compounds [RbSCN(dibenzo-30-crown-10) monohydrate and KSCN(dibenzo-30-crown-10) monohydrate], which have well characterised crystal structures and apparently identical carbon skeletons, show differences in multiplicity which cannot be easily interpreted. It comes as no surprise therefore that the interpretation of spectra of a much more-complex material such as native cellulose is the subject of some discussion, given that there is still controversy even amongst crystallographers regarding the structure of cellulose I. Obtaining spectra of *Valonia* cellulose at 7 T and using the Gaussian multiplication technique to enhance the data resolution yields some extra information (Fig. 2). For example, the smallest of the three peaks assigned to C-4 in the spectrum of *Valonia* cellulose is asymmetric, having a low-frequency shoulder. We believe that these features are real in that they occur reproducibly following resolution enhancement in a number of *Valonia* cellulose spectra. They have not been previously reported. Spectra obtained at higher field-strengths and the use of more sophisticated resolution-enhancement techniques such as constrained deconvolution[19] and maximum entropy[20] may improve the

resolution still further. The results presented here do not discriminate between the hypotheses of Atalla and VanderHart[10] or of Cael *et al.*[11] concerning the detailed interpretation of spectra of cellulose I. Both of these studies suggest that the observed multiplicities in the C-1 and C-4 resonances of native cellulose arise from two different crystal environments. Detailed features such as the asymmetry of one component of the C-4 multiplet may be consistent with both hypotheses. Such features could arise from effects within one crystal environment[18] (that is, differences in crystal-packing forces or small differences in conformation). Alternatively, native cellulose could be made up of more than two crystal environments. The real problem is that there is an almost total lack of any quantitative theory of chemical shifts and multiplicites in solid-state n.m.r. Without such a theory, the problem of the structure of native cellulose will not be solved by the acquisition of conventional c.p.–m.a.s. ^{13}C-n.m.r. spectra

A final aspect of the foregoing study is the recording—we believe for the first time—of a ^{13}C-n.m.r. spectrum of cellulose III_I. The spectrum is shown in Fig 5 and consists of a number of relatively broad signals. This spectrum is quite different from spectra of the other cellulose polymorphs and we conclude therefore that c.p.–m.a.s. ^{13}C-n.m.r. is a useful technique in distinguishing between cellulose polymorphs.

Comparison of the C-6 chemical shifts of cellulose I and III_I (Table I) suggests that there may be differences between these polymorphs in the orientation of the respective hydroxyl groups around the C-5–C-6 bond. Horii *et al* [21] have proposed a correlation between the ^{13}C chemical shift of C-6 and the torsion angle χ (describing this orientation). This suggests that the *tg* conformation of cellulose I is converted into the *gt* conformation in cellulose III_I. Such an interpretation is supported if one considers the intermediate compound between cellulose I and cellulose III_I, that is, the highly crystalline ethylenediamine–cellulose complex. The crystal structure of this complex has been resolved with a great precision and a definite *gt* conformation has been found for the C-6 hydroxyl group[22]. Mild removal of the ethylenediamine with methanol at room temperature yields cellulose III_I, and under such mild conditions it is not unreasonable to suppose that the orientation at C-6 remains unchanged. Thus, one of the differences between cellulose I and cellulose III_I appears to be the orientation of the C-6 hydroxyl group. As this group is locked by ethylenediamine into the *gt* conformation, the recovery of the cellulose I lattice is not possible when the ethylenediamine is washed out To convert back to cellulose I, it is necessary to change from the *gt* to the *tg* conformation by overcoming the rotational energy-barrier of the hydrogen-bonded C-6 hydroxyl group. This is achieved by a rather severe heating treatment of cellulose III_I with water at 160° A word of caution is required, however, in that factors affecting ^{13}C chemical shifts in the solid state are poorly understood and no really successful theoretical study of solid-state ^{13}C-n.m.r. chemical shifts has yet appeared. Further, the correlation formulated by Horii *et al.*[21] between χ and the chemical shift of C-6 does not always hold for certain cyclodextrins[23,24] and

consequently the conclusion that the transition from native to cellulose III$_I$ is accompanied by a change in orientation at C-6 must remain tentative.

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