

as a consequence of small errors in the measurements of  $\langle\mu^2\rangle/nm^2$  that can affect in a significant way the value of the temperature coefficient.

## References and Notes

- (1) Flory, P. J. *Statistical Mechanics of Chain Molecules*; Wiley-Interscience: New York, 1969.
- (2) Abe, A.; Mark, J. E. *J. Am. Chem. Soc.* **1976**, *98*, 6468.
- (3) Riande, E. *J. Polym. Sci., Polym. Phys. Ed.* **1976**, *14*, 2231; *Makromol. Chem.* **1977**, *178*, 2001.
- (4) Riande, E.; Mark, J. E. *Macromolecules* **1978**, *11*, 956.
- (5) Saiz, E.; Riande, E.; Guzmán, J.; de Abajo, J. *J. Chem. Phys.* **1980**, *73*, 958.
- (6) Riande, E. *Makromol. Chem., Macromol. Symp.* **1986**, *2*, 179.
- (7) Riande, E.; Guzmán, J.; Saiz, E.; Tarazona, M. P. *J. Polym. Sci., Polym. Phys. Ed.* **1985**, *23*, 1031.
- (8) Riande, E.; Guzmán, J.; Saiz, E. *Polymer* **1981**, *21*, 461.
- (9) Kops, J.; Larsen, E.; Spangaard, H. *J. Polym. Sci., Polym. Symp.* **1976**, *56*, 91.
- (10) Guggenheim, E. *Trans. Faraday Soc.* **1949**, *45*, 714; **1951**, *47*, 573.
- (11) Smith, J. W. *Trans. Faraday Soc.* **1950**, *46*, 394.
- (12) McClellan, A. L. *Tables of Experimental Dipole Moments*; Raha Enterprises: El Cerrito, CA, 1974; Vol. II.
- (13) Pitzer, K. *Adv. Chem. Phys.* **1959**, *2*, 49.
- (14) Yoon, D. Y.; Suter, U. W.; Sundararajan, P. R.; Flory, P. J. *Macromolecules* **1975**, *8*, 784.

Registry No. PTOXN, 75995-49-2.

## High-Resolution Solid-State Carbon-13 Nuclear Magnetic Resonance Study of Chitin

Steven F. Tanner\*

AFRC Institute of Food Research, Norwich Laboratory, Colney Lane,  
Norwich NR4 7UA, U.K.

Henri Chanzy

Centre de Recherches sur les Macromolécules Végétales, CNRS, B.P. 53X,  
38041 Grenoble Cédex, France

Marc Vincendon and Jean Claude Roux

Département de Recherches Fondamentales, Centre d'Etudes Nucléaires de Grenoble,  
B.P. 85X, 38041 Grenoble Cédex, France

Françoise Gaill

Centre de Biologie Cellulaire, CNRS, 67, rue Maurice Günsbourg, 94200 Ivry sur Seine,  
France

Received September 27, 1989

**ABSTRACT:** High-resolution  $^{13}\text{C}$  cross-polarization magic-angle spinning (CPMAS) NMR spectra have been acquired from a variety of solid chitin samples. Spectra of highly crystalline  $\beta$ -chitin contain peaks that have remarkably narrow line widths. Single lines can be assigned to all the chemically distinct carbon atoms of the chitin repeat, except for the carbonyl and the C2, which give rise to asymmetric doublets. These doublets arise from the failure of magic-angle spinning (MAS) to remove the dipolar interactions occurring between these carbon atoms and the directly bonded quadrupolar  $^{14}\text{N}$  nucleus. The single lines, assigned to the remaining carbon atoms, are consistent with a proposed crystal structure of the  $\beta$  polymorph. Spectra from at least three different forms of  $\beta$ -chitin have been identified. The differences between these three forms are a consequence of the differing states of hydration of the  $\beta$  polymorph. Spectra of several highly crystalline forms of  $\beta$ -chitin serve as reference spectra when interpreting the more complex spectra acquired from less ordered samples. The spectra of  $\alpha$ -chitin, presented here, contain broad lines and show distinct peak asymmetries. This makes it difficult to interpret spectra, to measure accurate values of chemical shifts, and to make useful comparisons between spectra of the  $\alpha$ - and  $\beta$ -chitin polymorphs.  $^{13}\text{C}$  relaxation rates for the C6 carbon are large relative to the other chemically distinct carbon atoms in chitin of low crystallinity. This difference in relaxation rate is particularly marked in  $\alpha$ -chitin.

## Introduction

$^{13}\text{C}$  cross-polarization magic-angle spinning (CPMAS) NMR has proved useful in studies of the structure and packing of molecules in the solid state.<sup>1-5</sup> A good example of the use of these techniques is provided by two crystalline native cellulose samples, *Valonia* and tunicin, which gave

distinct NMR spectra.<sup>6,7</sup>  $^{13}\text{C}$  CPMAS spectra are also useful in studying samples that are either heterogeneous or poorly crystalline since NMR signals can be observed from both crystalline and amorphous domains. The appearance of starch spectra, for example, depends on the degree of sample crystallinity and on the nature of the starch polymorph.<sup>8</sup> In this study we have used  $^{13}\text{C}$  CPMAS spectroscopy together with X-ray diffraction to investigate the structure of a number of chitin samples of

\* To whom all correspondence should be addressed.

different origin and varying crystallinity.

Chitin consists of  $\beta$ -1-4-linked residues of 2-deoxy-2-acetamido-D-glucose. This polysaccharide is similar to cellulose therefore, differing only in that it contains an *N*-acetylamido instead of a hydroxyl group at C2. Solid chitin occurs in nature in two principal polymorphic forms that are readily distinguished on the basis of their different infrared spectra and X-ray diffraction patterns.<sup>9-12</sup>  $\alpha$ -Chitin is the most stable and the most abundant polymorph. It is found chiefly in the exoskeleton of arthropods and in certain fungi. The unit cell in crystalline  $\alpha$ -chitin is thought to be characterized by a  $P2_12_12_1$  space group and to contain two antiparallel chains.<sup>13,14</sup>  $\beta$ -Chitin occurs in squid pen, in pogonophore tube, in the spines of certain diatoms, and in *Aphrodite chaetae*. Crystalline  $\beta$ -chitin has a monoclinic unit cell with  $P2_1$  symmetry and a single chain located on the  $2_1$  axis.<sup>15-18</sup> The chitin chains in this polymorph are therefore packed in parallel. Structural studies have proposed that  $\beta$ -chitin adopts sheetlike structures that are formed by hydrogen bonds linking the  $\text{CH}_2\text{OH}$  with the carbonyl groups on neighboring chains. When  $\beta$ -chitin swells in water, these sheets remain intact but move apart to include the water molecules.<sup>17</sup>  $\beta$ -Chitin is metastable and can be converted into the  $\alpha$  form by solid-state swelling by using either hydrochloric acid or an aqueous solution of lithium thiocyanate.<sup>9,12</sup>

In this report we present  $^{13}\text{C}$  CPMAS spectra of some  $\beta$ -chitin hydrates and some  $\alpha$ -chitins and the spectra of some  $\beta$ -chitins that have been converted into the  $\alpha$  polymorph by a solid-state swelling process. Although  $^{13}\text{C}$  CPMAS spectra of chitin have been presented before,<sup>19-26</sup> the published spectra have been acquired from samples of rather low crystallinity and they therefore contain rather broad lines. Further, spectra of chitin samples that are not highly crystalline can show asymmetric peaks and distinct "shoulders". The combination of shoulders on broad peaks makes it difficult to obtain accurate chemical shift values for samples existing in defined states of packing. This is a problem of spectral interpretation that we have addressed in this study by acquiring the spectra of some very highly crystalline  $\beta$ -chitin samples that contain narrow lines. The acquisition of such spectra makes it easier to observe any peak multiplicities that might arise from the way in which chitin chains pack in the lattice. It also facilitates the interpretation of the more complex spectra acquired from chitin samples that show some degree of packing disorder. Further, we can compare spectra of highly crystalline chitin with the spectra obtained from cellulose, a polysaccharide that is structurally similar to chitin.

## Experimental Section

**Chitin Samples. Lobster Tendon  $\alpha$ -Chitin.** Small fragments of lobster tendon were incubated with pronase at pH 7 and 38 °C for 96 h to remove protein. The fragments were then washed in distilled water and freeze dried.

**Crab Shell  $\alpha$ -Chitin.** Crab shells were demineralized by treating them overnight in 1 N HCl. After neutralization, the protein was removed in a 2-h treatment under nitrogen using 1 N NaOH at 100 °C. The shells were then washed in distilled water and air dried prior to use.

**Squid Pen  $\beta$ -Chitin.** Protein was removed from fresh squid pen samples in a 2-h treatment in 1 N NaOH at 100 °C under nitrogen. The pens were then washed and freeze-dried. In an alternative preparation, protein was removed from the pens by incubating them with pronase at 38 °C and pH 7 for 96 h.

**Diatom Spine  $\beta$ -Chitin.** Cultures of the diatom *Thalassiosira fluviatilis* were grown and their chitin spines extracted and purified using the same methods developed by McLachlan et al.<sup>27</sup>

The cultures, in suspension, were subjected to a 5-s treatment in a Waring blender to loosen the spines from the diatom cells. Separation of spines from the walls occurred on centrifugation of the suspension at 4000 rpm for 30 min. The spines were filtered by using a 1.2- $\mu\text{m}$  Millipore filter, washed, redispersed, and subsequently subjected to a pronase treatment at pH 7 and 38 °C for 4 days. The spines were then filtered and immersed in a solution of 1% HF for 10 min at room temperature to remove any remaining silica. The spines were then thoroughly washed and finally air-dried. Approximately 100 mg of purified chitin spines was collected over a 3-month period.

**$\beta$ -Chitin from the Tubes of *Tevnia jerichonana*.** *T. jerichonana* is a vestimentiferan worm<sup>28</sup> that secretes a tube consisting of chitin and protein. These worms, considered previously as pogonopheran, are now placed in the putative phylum Vestimentifera. They are found at a depth of 2600 m around deep-sea hydrothermal vents where the vent fluid mixes with surrounding sea water. The tubes used in this study were collected by the submersible Nautille during the Hydronaut cruise (November 1987). The tubes were harvested at the site located at 12°48' N, 103°56' W. The tubes were preserved in ethanol or fixed in formal saline mixtures. For some NMR experiments, a sample of *Tevnia* tubes was dried in vacuo for 70 h at 60 °C, whereas other samples were studied at ambient humidity (the so-called hydrated *Tevnia* tube samples). For studies using electron microscopy, pieces of tube were postfixed with osmium tetroxide (1% final concentration) and embedded in Durcupan. Thin sections were stained with aqueous uranyl acetate and lead citrate prior to examination with a Philips EM201 operated at 80 kV.

**Solid-State Transformation of  $\beta$ - into  $\alpha$ -Chitin.** Squid pen  $\beta$ -chitin was converted into the  $\alpha$  polymorph by soaking pen fragments in 6 M HCl for 6 h at room temperature. The fragments were then neutralized, washed thoroughly, and air-dried. In an alternative procedure, the  $\beta$  to  $\alpha$  conversion was achieved by immersing the squid pens in a saturated aqueous LiSCN solution for 8 h at 100 °C.

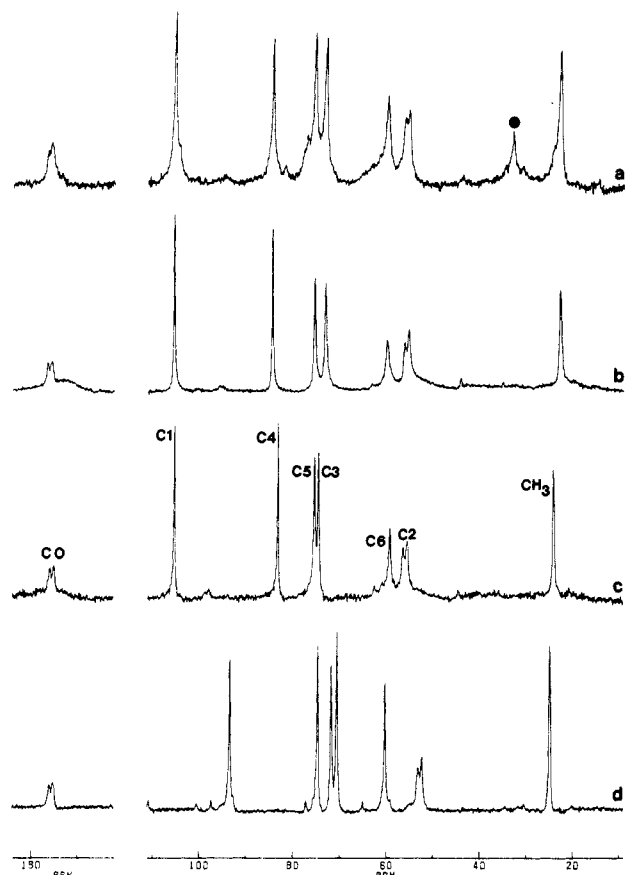
***N*-Acetyl-D-glucosamine** was purchased from Sigma and used without further purification.

**NMR Spectroscopy.** Most of the NMR measurements were carried out with a Bruker CXP 300 spectrometer operating at a magnetic field strength ( $B_0$ ) of 7.05 T. Spectra were acquired with cross-polarization magic-angle spinning (CPMAS) techniques with a single contact pulse of 1-ms duration, a spectral width of 17 kHz, and recovery times between acquisitions of at least 5 s. Proton decoupling fields of ca. 65 kHz were employed in these experiments together with modest spinning rates of ca. 3 kHz.  $^{13}\text{C}$  chemical shifts are quoted with respect to tetramethylsilane (TMS) and were obtained relative to the shifts of adamantane (assumed to be 38.56 and 29.51 ppm from TMS<sup>29</sup>), which was used as the secondary external reference. The adamantane shift was measured prior and subsequent to each acquisition of interest. VanderHart has shown that the measured chemical shifts of certain solids depend on the strength of the applied magnetic field ( $B_0$ ).<sup>30</sup> Consequently, errors can occur in quoted shift values, if, for example, linear polyethylene is used as the reference standard. This is not such a serious problem when adamantane is used as the secondary reference, particularly at the relatively high  $B_0$  field used here. One NMR spectrum was acquired using an NMR spectrometer operating at a  $B_0$  field strength of 5 T.

**X-ray Analysis.** For the *Tevnia* tube, X-ray experiments were carried out with a Philips PW1820 goniometer and an attached Anton Paar TTK vacuum camera. For the hydrated sample, the camera was flushed continuously with wet helium while the dried samples were examined in a sealed camera containing silica gel. The squid pen samples were analyzed with a Siemens Kristalloflex generator equipped with a Wahren vacuum camera.

## Results and Discussion

**Highly Crystalline  $\beta$ -Chitin.**  $^{13}\text{C}$  CPMAS spectra of some  $\beta$ -chitins together with the spectrum of *N*-acetyl-D-glucosamine are shown in Figure 1. Figure 1a is the spectrum of diatom spines isolated from *Thalassiosira fluviatilis* while parts b and c of Figure 1 are spectra of dried



**Figure 1.** Solid-state  $^{13}\text{C}$  CPMAS spectra of (a) diatom spines isolated from *Thalassiosira fluviatilis*, (b) the dried tube of a vestimentiferan worm (*Tevnia jerichonana*), (c) the hydrated tube of the vestimentiferan worm. (a)–(c) are spectra of highly crystalline  $\beta$ -chitin. The peak labeled (●) in (a) is due to protein. Figure 1d is the  $^{13}\text{C}$  CPMAS spectrum of *N*-acetyl-D-glucosamine.

and hydrated samples of *Tevnia jerichonana* tube. Line widths in these spectra are remarkably small. For example, in Figure 1c, the lines are approximately 20-Hz wide, which is comparable to line widths observed in certain spectra of small and highly crystalline organic molecules. Thus line widths are similar in the spectra of hydrated *Tevnia* and *N*-acetyl-D-glucosamine (parts c and d of Figure 1), indicating that the polysaccharide sample is very highly crystalline. This is confirmed by the X-ray diffraction patterns of the *Tevnia* samples (Figure 2). Comparison of these *Tevnia* tube diffraction patterns with those obtained by Blackwell from pogonophore tube  $\beta$ -chitin<sup>17</sup> shows that the hydrated *Tevnia* tube, with a reflection at  $d = 10.9 \text{ \AA}$ , is the higher hydrated form of  $\beta$ -chitin, whereas the dried sample ( $d = 9.14 \text{ \AA}$ ) is the crystalline anhydrous form.

Single lines can be assigned in each spectrum of Figure 1<sup>31</sup> to six of the eight chemically distinct carbon atoms of the 2-deoxy-2-acetamido-D-glucose repeat unit. The C2 and the carbonyl carbons, however, give rise to asymmetric doublets with a splitting of ca. 60–70 Hz (see Table I). These splittings arise from the failure of magic-angle spinning (MAS) to remove the  $^{13}\text{C}$ – $^{14}\text{N}$  dipolar interactions for the two carbon atoms that are directly bonded to the quadrupolar  $^{14}\text{N}$  nucleus of the acetamido group.<sup>32,33</sup> The magnitude of each splitting depends on a number of parameters,<sup>32</sup> which include the strength of the applied magnetic field ( $B_0$ ) and the size of the  $^{14}\text{N}$  quadrupole coupling constant ( $\chi$ ). Acquiring a spectrum at lower field (5 T) results in a larger splitting (ca. 100 Hz) at both the C2 and the carbonyl (see Table I). This observation

**Table I**  
 **$^{13}\text{C}$  Chemical Shifts of Solid Chitin Samples**

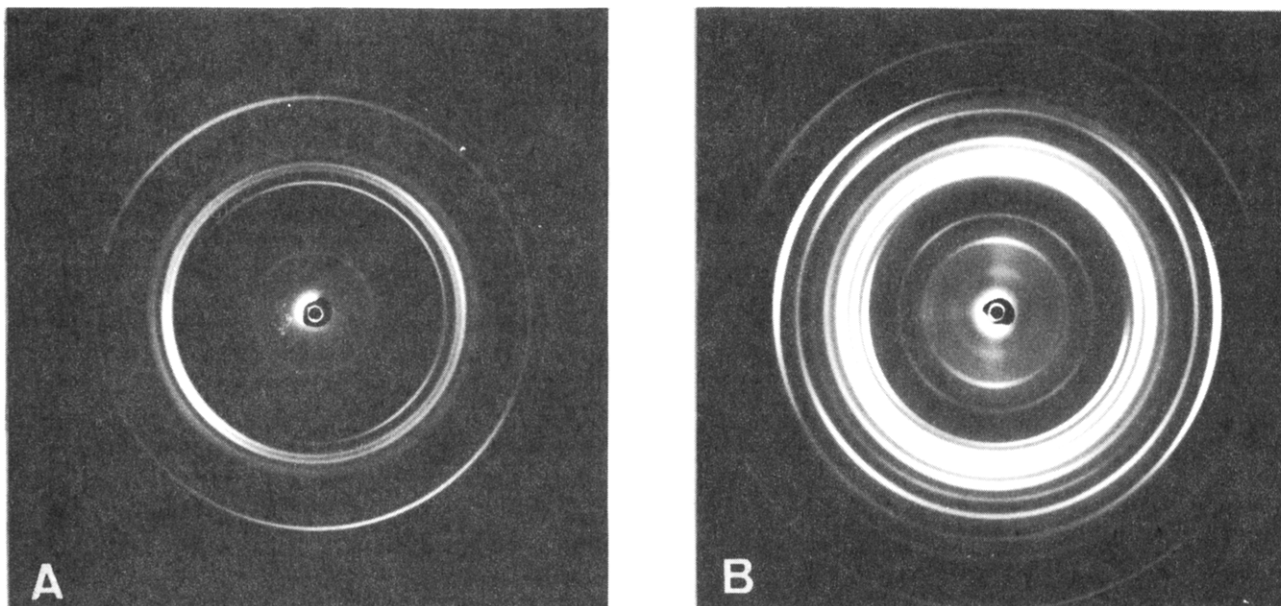
	$\beta$ chitin				$\alpha$ chitin	
	diatom spines	<i>Tevnia</i> tube		wet squid pen	lobster tendon	crab shell
		dried	hydrated <sup>a</sup>			
C1	105.4	105.3	105.2	104.8	104.6	104.5
C2	55.3	55.2	55.5(55.2)	56.0	55.6	55.4
	56.1	56.0	56.4(57.1)			
C3	73.1	73.1	74.5	74.5	73.7	73.6
C4	84.5	84.4	83.1	83.7	83.6	83.2
C5	75.5	75.4	75.4	75.4	76.0	76.0
C6	59.9	59.8	59.2	59.3	61.1	61.0
				61.0		
C=O	175.6	175.5	175.0(174.5)	174.8	173.0	173.7
	176.4	176.4	175.8(176.4)			
CH <sub>3</sub>	22.8	22.7	24.1	23.3	23.1	23.1
				24.1		

<sup>a</sup> Chemical shifts shown in parentheses were acquired at a  $B_0$  field strength of 5 T.

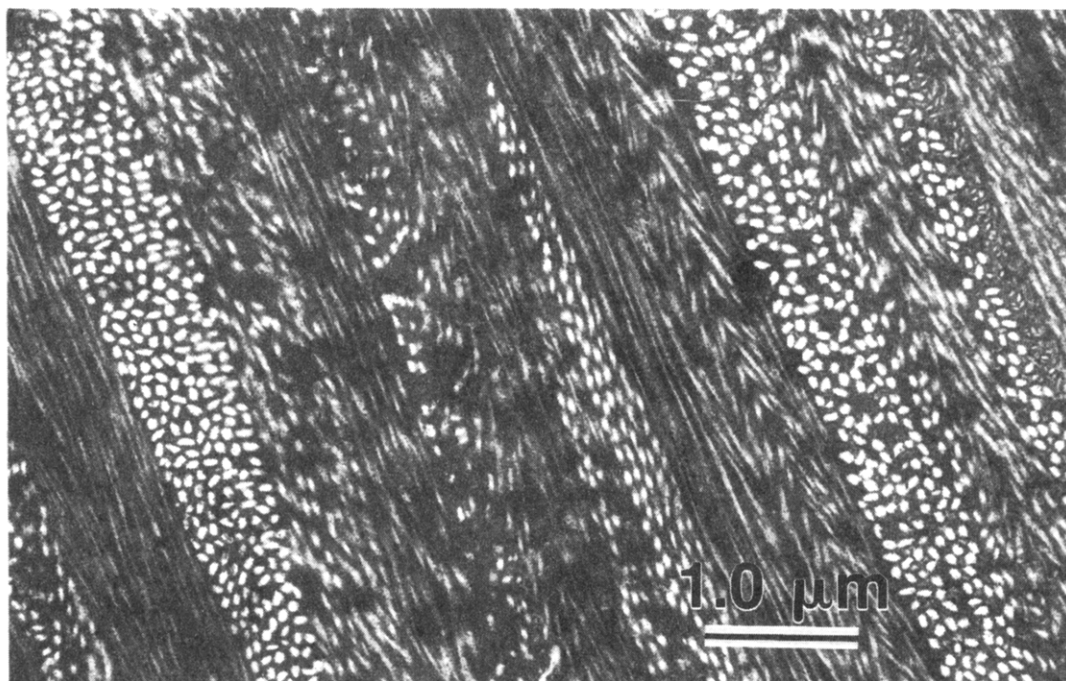
confirms our hypothesis as to the origin of these splittings. The asymmetric doublets seen in Figure 1 are a little unusual in that such splittings have only rarely been observed before at the relatively high field strengths used in this study (7.04 T).<sup>34</sup> The observation of such splittings is due mainly to the narrow lines occurring in these spectra and to the value of  $\chi$  for  $^{14}\text{N}$  in the acetamido group which is probably relatively large (ca.  $-3 \text{ MHz}$ ).<sup>32,35</sup>

The unit cell of anhydrous  $\beta$ -chitin is believed to contain a single chain with  $P2_1$  symmetry and a chitobiosyl repeat unit.<sup>11</sup> The observation of single lines for each carbon atom of the 2-deoxy-2-acetamido-D-glucose unit in the dried *Tevnia* tube spectrum (apart from the resonances assigned to C2 and the carbonyl) is consistent with this proposed crystal structure.

Close examination of Figure 1 (and Table I) reveals that there are slight differences between the spectra of dried (anhydrous) and hydrated *Tevnia* tubes. The spectrum of the hydrated sample (the higher hydrate) contains some peaks that are shifted relative to those in the spectrum of anhydrous  $\beta$ -chitin. This, together with the X-ray diffraction patterns of these samples, indicates that the packing of the polymer chains differs in these two forms of  $\beta$ -chitin. The changes in both the NMR and the X-ray diffraction patterns resulting from sample dehydration are easily reversed. Merely soaking the dried sample in water gives a material that has a spectrum identical with that shown in Figure 1c. The addition of water to anhydrous  $\beta$ -chitin results in an increase in  $d$  spacing from 9.14 to 10–11  $\text{\AA}$  (Figure 2). Blackwell has observed this previously<sup>17</sup> in his studies of the structure of  $\beta$ -chitin obtained from a pogonophore tube. He relates this increase in  $d$  spacing to water entering between, and hence forcing apart,  $\beta$ -chitin sheets formed by a number of intrasheet hydrogen bonds. The chemical shifts of C1 and C4 carbons in 1–4-linked carbohydrates are believed to be highly sensitive to the conformation of the glycosidic linkage.<sup>36–38</sup> The difference in the C4 shifts between anhydrous and hydrated  $\beta$ -chitins is only 1.3 ppm, while at C1, it is even less (Table I). This suggests that any differences in the conformations of the glycosidic linkages in these two samples are small. Although crystal structures of hydrated  $\beta$ -chitins have not been determined, it is known that the addition of water to the anhydrous form results in an increase in only one of the dimensions of the unit cell. The volume of the unit cell of the hydrate is such that it can only contain a single polymer chain. This observation also suggests that water of crystallization does not dramatically change the packing of the chitin chains. Further, the C6



**Figure 2.** X-ray diffraction patterns obtained from (A) the hydrated tube of the ventimentiferan worm *Tevnia jerichonana* and (B) the dried tube of the vestimentiferan worm.

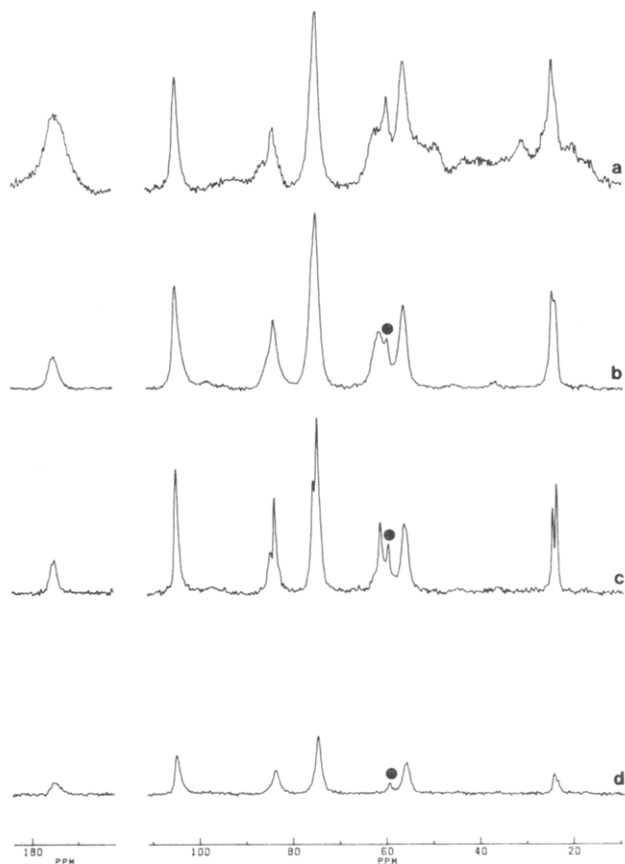


**Figure 3.** Electron micrograph of the cross section of the tube of *Tevnia jerichonana*. The section was stained with uranyl acetate and lead citrate. The white areas correspond to the  $\beta$ -chitin crystals and the dark areas to the protein matrix.

chemical shift in all samples of  $\beta$ -chitin falls in the range of 59.2–61.0 ppm. Horii et al.<sup>39</sup> have suggested that a correlation exists between the C6 shift and the conformation of the primary hydroxyl group. The use of this correlation indicates that C6 adopts a gg conformation in both anhydrous and hydrated  $\beta$ -chitins. This is in good agreement with the proposed crystal structure of the anhydrous form. We would stress, however, that this method of using  $^{13}\text{C}$  chemical shifts to determine the C6 conformation is not universally applicable.<sup>40</sup>

An electron micrograph of a *Tevnia* tube (Figure 3) shows that this sample almost certainly consists of blocks of highly crystalline chitin in an amorphous matrix. Rudall<sup>41</sup> has shown that most chitin protein complexes, stained as in this study, give micrographs where the chitin appears white and the protein black (compare Figure 3).

Diffraction contrast electron microscopy carried out on *Tevnia*<sup>42</sup> shows that the white areas consist of chitin in that they are susceptible to diffraction contrast whereas the black areas are not, irrespective of sample orientation. Further, Gaill et al.<sup>43</sup> have carried out protein analysis on tubes similar to *Tevnia*. In spite of varying the spectral acquisition conditions (i.e., the contact and recycle times), we were unable to obtain any evidence for protein peaks in cross-polarization spectra of a *Tevnia* tube.<sup>44</sup> Attempts at "seeing" protein by using the so-called single-pulse excitation pulse sequence<sup>45</sup> also failed. This is in marked contrast to spectra of squid pen (Figure 4a) and also  $\alpha$ -chitins obtained from crab and lobster (not shown) where protein peaks are detected. We cannot explain the absence of protein peaks in parts b and c of Figure 1. Their absence represents an extreme example of a cross-polarization

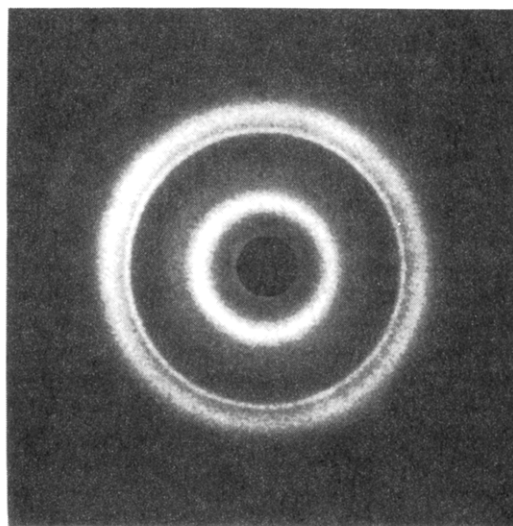


**Figure 4.**  $^{13}\text{C}$  CPMAS spectra of (a) a whole squid pen (this spectrum contains peaks that can be assigned to both  $\beta$ -chitin and protein), (b) squid pen  $\beta$ -chitin, (c) squid pen  $\beta$ -chitin soaked in water, (d) squid pen  $\beta$ -chitin obtained using a pulse sequence designed to measure  $^{13}\text{C}$   $T_1$  values. A  $\tau$  (delay) value of 20 s was used in this sequence. Spectra (b) and (d) have been plotted with the same absolute intensity calibrations such that differences in the peak intensities between these spectra reflect the spin-lattice relaxation. The peaks marked (●) denote the C6 signal with the longer  $T_1$  value (see text).

spectrum in which peak areas do not reflect the concentrations of  $^{13}\text{C}$  nuclei in the sample.

The spectra of  $\beta$ -chitin, shown in Figure 1, have been obtained from some very highly crystalline samples. The line widths in the spectrum of a hydrated *Tevnia* tube are narrower (ca. 20 Hz) than those in the corresponding spectrum of *Valonia ventricosa* (ca. 40 Hz),<sup>7</sup> a very highly crystalline sample of native cellulose. This provides further evidence for the perfection of the *Tevnia* tube crystallites. The spectra in Figure 1 are useful in interpreting NMR spectra of less highly ordered  $\beta$ -chitins, which give spectra containing broad and asymmetric peaks.

**Squid Pen  $\beta$ -Chitin.** Figure 4a shows a  $^{13}\text{C}$  CPMAS spectrum of solid squid pen. This spectrum contains peaks that can be assigned to both the carbohydrate and protein components of this sample. The spectrum of squid pen in which protein has been removed (in a solid-state treatment using pronase) contains a number of features of interest. This spectrum (Figure 4b) is characterized by broad asymmetric peaks and by distinct shoulders at C6 and the methyl resonance. These spectral features make it difficult to obtain accurate and reproducible values of chemical shifts. The spectrum shown in Figure 4b is similar to chitin spectra reported previously<sup>22–26</sup> in that the line widths are relatively large. The X-ray diffraction pattern of squid pen  $\beta$ -chitin, shown in Figure 5, reveals that this sample is poorly crystalline. This is a major source of the broad lines (ca. 150 Hz) seen in Figure 4b. The width



**Figure 5.** X-ray diffraction patterns of squid pen  $\beta$ -chitin. The broad lines correspond to the equatorial diffractions and the sharp lines to the meridional diffractions. This denotes the presence of long and very narrow crystals of  $\beta$ -chitin in squid pen.

of the squid pen microfibrils (i.e., the lateral width of the crystallites) is only 2–3 nm. This implies that, in squid pen chitin, a significant proportion of the polysaccharide chains are found on the surface of the crystallites with relatively few chains occurring within crystallite interiors. Although there are a number of line-broadening mechanisms in solid-state  $^{13}\text{C}$  CPMAS NMR,<sup>46</sup> previous studies on native cellulose<sup>6</sup> have assigned broad lines to chains existing either in regions of packing disorder or at the surface of crystallites while narrow lines arise from chains inside the more crystalline parts of the cellulose sample. These observations are consistent with those in this study. Chitin samples that are shown by X-ray diffraction to be highly crystalline give rise to very narrow lines in their NMR spectra while the less crystalline samples give broader NMR peaks. The peaks in Figure 4 are heterogeneously broadened to an extent that any splittings of C2 and the carbonyl peaks (such as those observed in Figure 1) are not resolved.

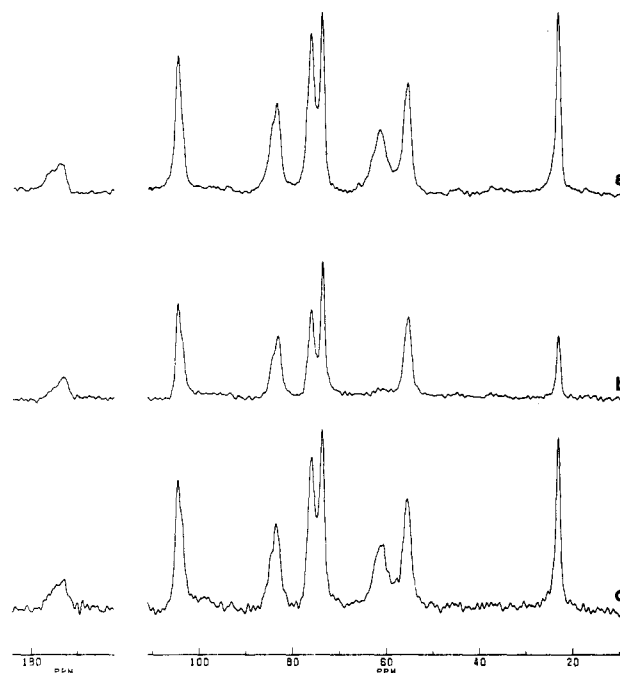
Parts b and c of Figure 4 show that line widths in the  $^{13}\text{C}$  CPMAS spectra of squid pen chitin are sensitive to water content. Samples that contain "excess" water (that is, they contain free or freezable water) give spectra (Figure 4c) containing relatively narrow lines. This reflects an increase in sample crystallinity on wetting. Although water has improved the resolution in squid pen spectra, the line widths in Figure 4c are still greater than those observed in the spectra of a *Tevnia* tube. It is not known why water improves the crystallinity in  $\beta$ -chitin; however, in starch and paramylon,<sup>40</sup> it is thought that water facilitates chain motion, thus removing strain and hence removing defects within the sample. The CPMAS experiment can cause water loss,<sup>40</sup> which reduces crystallinity and therefore introduces some variability in the spectral appearance.

Examination of Figure 4c reveals that certain carbon atoms give rise to peaks that consist of at least two major components. This is most obvious for resonances assigned to the methyl group where two partially resolved lines are observed at 23.3 and 24.1 ppm from TMS. Comparison of Figures 4c and 1c shows that the spectrum of wet squid pen chitin contains all the resonances of the hydrated *Tevnia* tube spectrum in addition to some extra peaks. The spectrum of a hydrated *Tevnia* tube is therefore a "subset" of the squid pen spectrum. For example, both squid pen and *Tevnia* have methyl peaks at 24.1 ppm, but only the



wet squid pen spectrum contains a second methyl peak at 23.3 ppm (Table I). Further the C1 peak in *Tevnia* is completely contained within the envelope of the squid pen C1 signal. These "extra" peaks in the wet squid pen spectrum reflect the presence of chitin chains that pack in at least two distinct ways in this sample. Supporting evidence for this hypothesis is provided in Figure 4d, which shows the spectrum of squid pen chitin obtained by using a pulse sequence, developed by Torchia,<sup>47</sup> to measure  $^{13}\text{C}$  spin-lattice relaxation times ( $T_1$ ). Comparison of parts b and d of Figure 4 shows that when a  $\tau$  value of 20 s is used in the  $T_1$  sequence, the high-frequency component of the C6 line (at 61.0 ppm) disappears from the squid pen spectrum, leaving only the C6 component at 59.3 ppm (marked with a dot). Thus the two C6 components in Figure 4c have very different  $^{13}\text{C}$   $T_1$  values, an observation that almost certainly indicates that these two components arise from distinctly different environments within this sample. The C6 peak with the longer  $^{13}\text{C}$   $T_1$  value in the squid pen spectrum (marked with a dot) has a chemical shift identical with the single C6 line in the spectrum of a hydrated *Tevnia* tube. This peak is assigned to the primary hydroxyl carbon of a crystalline hydrate form of  $\beta$ -chitin. The relatively long  $T_1$  of this line may therefore reflect some hindered mobility due to the involvement of the primary hydroxyl group in the hydrogen bonding that forms the sheet structure in crystalline  $\beta$ -chitin.<sup>17</sup> Measurement of  $^{13}\text{C}$  spin-lattice relaxation times of a hydrated *Tevnia* tube gave values of between 110 and 160 s for the ring carbons, 50 s for the methyl and ca. 30 s for C6. These are considerably longer than the corresponding  $T_1$  values in squid pen chitin. The spin-lattice relaxation times of peaks in the squid pen spectra are not quoted since they were found to be irreproducible. This may be a consequence of water loss during the CPMAS experiment. Although the  $^{13}\text{C}$  spin-lattice relaxation times in squid pen are somewhat irreproducible, the  $T_1$  values of the C6 line at 61.0 ppm were always shorter than those at 59.3 ppm. Given that the spectra of anhydrous crystalline  $\beta$ -chitin (parts a and b of Figure 1) contain resonances with chemical shifts different from those in the spectrum of wet squid pen chitin, it is clear that we have identified at least three forms of  $\beta$ -chitin in this study (Table I). These are a crystalline anhydrous form (methyl resonance at 22.7 ppm), a crystalline (higher) hydrate (methyl resonance at 24.1 ppm), and a third, as yet unidentified, component (23.3 ppm). This third form could arise from chitin chains existing at the surface of crystallites or in regions of packing disorder or it could represent a second chitin hydrate. The latter possibility seems more likely, since, first, all lines in Figure 4c are relatively narrow and, second, at least two hydrate forms of  $\beta$ -chitin have been reported previously.<sup>17</sup> The  $^{13}\text{C}$  CPMAS spectra of crystalline samples of  $\beta$ -chitin and native cellulose therefore show that both polysaccharides are capable of existing in a number of different forms. For native cellulose, NMR results suggest that any sample can be made up of two allomorphs (the  $I\alpha$  and  $I\beta$  forms). Differences between these allomorphs are believed to be small and are thought to relate to slight differences in hydrogen bonding. In  $\beta$ -chitin, differences between at least two of the three forms identified here by using NMR are associated with water content. The origin of these differences may be a consequence of variations in the hydrogen bonding occurring between chitin chains and the water of crystallization.

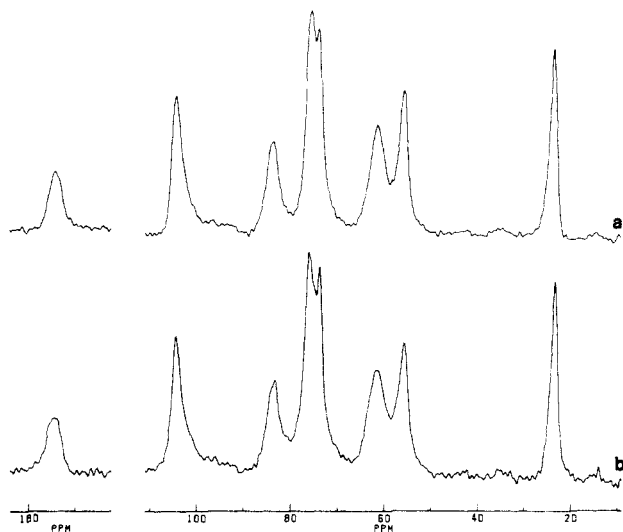
**$\alpha$ -Chitin.** The  $^{13}\text{C}$  spectrum of lobster and crab shell  $\alpha$ -chitin together with the spectrum of crab shell chitin acquired using the  $T_1$  pulse sequence is displayed in Figure



**Figure 6.**  $^{13}\text{C}$  CPMAS spectra of solid  $\alpha$ -chitin: (a) crab shell  $\alpha$ -chitin, (b) crab shell  $\alpha$ -chitin acquired by using a pulse sequence designed to measure  $^{13}\text{C}$   $T_1$  (using a 20-s  $\tau$  delay). Spectra (a) and (b) have been plotted with the same absolute intensity calibrations such that differences in the peak intensities between these spectra reflect the spin-lattice relaxation. Figure 6c is the  $^{13}\text{C}$  CPMAS spectrum of solid lobster  $\alpha$ -chitin.

6. The lines in these spectra are broad, indicating that these  $\alpha$ -chitin samples are poorly crystalline.<sup>19-21,23-25</sup> The peaks in Figure 6 are also distinctly asymmetric. Comparison of these spectra with the squid pen  $\beta$ -chitin spectrum shows that the C3 and C5 carbon atoms give two partially resolved peaks for  $\alpha$ -chitin but only a single broad and asymmetric peak for the  $\beta$  polymorph. At first glance, therefore, an obvious spectroscopic method of separating these chitin polymorphs would be based upon the C3 and C5 chemical shifts (Table I). Close examination of the spectrum of anhydrous  $\beta$ -chitin, however (parts a and b of Figure 1), reveals that the chemical shift difference between C3 and C5 is also relatively large for this form of  $\beta$ -chitin. In this respect, the spectra of  $\alpha$ -chitin and the anhydrous form of the  $\beta$  polymorph are similar. The use of the C3 and C5 chemical shifts is, therefore, not a reliable method for identifying chitin polymorphs, particularly when information relating to sample hydration is unavailable.

The spectrum of crab shell chitin obtained by using a value of 20 s for the  $\tau$  delay in the  $T_1$  pulse sequence (Figure 6b) does not contain a C6 peak. Thus the C6  $T_1$  is very short in comparison with the  $T_1$ s of the other carbon atoms of the 2-deoxy-2-acetamido-D-glucose repeat. This increased C6 spin-lattice relaxation rate is probably a consequence of the rapid motion of the pendant  $\text{CH}_2\text{OH}$  group and of the two directly bonded protons on C6 which increases the efficiency of dipolar relaxation relative to the other carbons in the sugar ring. The use of the  $T_1$  pulse sequence gives somewhat different results for crab shell and squid pen chitins. For the hydrated  $\beta$  polymorph, the use of this pulse program shows that the chitin chains pack in at least two distinct ways. For the  $\alpha$  form the use of the  $T_1$  pulse program appears to decrease peak intensity while leaving the line shapes unaltered. Comparison of Figures 4 and 6 shows that one difference between the less crystalline (and more common) forms of  $\alpha$ - and  $\beta$ -chitin is the relaxation rate of C6 relative to the other carbon



**Figure 7.**  $^{13}\text{C}$  CPMAS spectra of  $\alpha$ -chitin obtained by the solid-state conversion of squid pen  $\beta$ -chitin by using (a) hydrochloric acid (b) a hot, saturated solution of lithium thiocyanate.

atoms of the 2-deoxy-2-acetamido-D-glucose unit. For  $\alpha$ -chitin this difference in relaxation rate is much greater than that observed for squid pen  $\beta$ -chitin, which has one C6 component with a relatively long spin-lattice relaxation time.

The interpretation of the squid pen  $\beta$ -chitin spectrum was facilitated by having access to the spectrum of a highly crystalline sample of this polymorph (i.e., chitin from a *Tevnia* tube). Unfortunately, we do not possess such a highly crystalline sample of  $\alpha$ -chitin, and consequently we are not able to interpret spectral features, such as peak asymmetries, seen in Figure 6. Further, because the chemical shift difference for a given carbon atom in the spectra of  $\alpha$ - and  $\beta$ -chitin is less than 2 ppm (Table I), it is difficult to make useful comparisons between the spectra of these polymorphs especially when those spectra contain broad and asymmetric lines. These problems are compounded when comparing chemical shifts measured on spectrometers operating at different frequencies or spectra referenced to a number of secondary external standards.<sup>30</sup>

**Conversion of  $\beta$ -Chitin into the  $\alpha$  Polymorph.** The solid-state chemical conversion of  $\beta$ -chitin into the more stable  $\alpha$  polymorph can be monitored using  $^{13}\text{C}$  CPMAS NMR. Figure 7 contains spectra of squid pen chitin subsequent to treatment using either hydrochloric acid or a hot, saturated solution of lithium thiocyanate. During this chemical conversion the chitin remains in the solid phase. Although one must be cautious in using the NMR chemical shifts obtained from Figure 7, these data together with the X-ray diffraction patterns show that the original  $\beta$ -chitin samples have been converted into the  $\alpha$  polymorph. Line widths in Figure 7 are greater than those observed in Figures 4b and 6, suggesting that this chemical transformation has caused an increase in the amount of disordered material in these samples.

## Conclusion

The spectra of highly crystalline  $\beta$ -chitin presented here serve as reference spectra as they have been used to interpret the more complex spectra acquired from less crystalline samples. Spectra of highly ordered  $\beta$ -chitin contain very narrow lines. Single peaks have been assigned to six of the eight chemically distinct carbon atoms of the *N*-acetylglucosamine repeat. The C2 and the carbonyl give rise to asymmetric doublets however, due to the failure

of MAS to remove the dipole-dipole interactions occurring between these carbon atoms and the directly bonded quadrupolar  $^{14}\text{N}$  nucleus. The observation of single lines for the remaining carbon atoms is in agreement with the structure determinations of Blackwell et al., who propose a chitobiosyl repeat and a unit cell containing a single chain located on a  $P2_1$  symmetry axis.<sup>11,17</sup> It does not agree with an alternative packing scheme for  $\beta$ -chitin that suggests a 4-fold increase in the length of the *b* axis and a unit cell containing four chitin chains.<sup>48</sup> We have cross-sectioned some *Tevnia* tube crystals and have obtained diffraction patterns from these sections. These patterns show spots that are consistent with a unit cell containing a single chain, there being no hint of an increase in size of the unit cell.<sup>42</sup> Chemical shift measurements of C6 in  $\beta$ -chitin spectra indicate that the primary hydroxyl adopts a *gg* conformation. This also agrees with Blackwell's proposed packing scheme. Single lines seen in the spectra of  $\beta$ -chitin may be contrasted with the spectra of native cellulose where peak multiplicities are observed and where some controversy exists in the interpretation of both NMR and diffraction data.

Spectral features associated with at least three different forms of  $\beta$ -chitin have been identified in this study. There are relatively small differences between spectra of the higher hydrate and the anhydrous form, suggesting that the water does not cause large changes in conformations about the glycosidic linkage. Water is, however, an important factor in affecting the structures and hence the spectra of a number of carbohydrates including  $\alpha$ -D-glucose,<sup>49</sup> starch,<sup>8</sup> paramylon,<sup>50,51</sup> and cyclodextrins.<sup>40</sup> The spectra of squid pen  $\beta$ -chitin, a relatively poorly ordered sample, were interpreted by using the spectra of highly crystalline samples of  $\beta$ -chitin.

Unfortunately we do not possess an equivalent highly ordered sample of the  $\alpha$  polymorph. The spectra of  $\alpha$ -chitin presented here therefore contain broad lines and are difficult to interpret. They also show distinct peak asymmetries. This causes problems in measuring accurate chemical shift values. Although there are differences in the chemical shifts between the  $\alpha$ - and  $\beta$ -chitin polymorphs, these differences are of the same order as the differences in chemical shifts between the various hydrate forms of  $\beta$ -chitin.

**Acknowledgment.** We thank Dr. A. M. Alayse-Danet, chief scientist of the Hydronaut cruise, for his help in collecting the *Tevnia* tubes and Dr. W. Herth for providing the initial cultures of *Thalassiosira fluviatilis*. We also acknowledge and thank P. Cairns (Institute of Food Research, Norwich, U.K.) for obtaining the diffraction patterns shown in Figure 2 and Dr. D. Williamson (University of East Anglia, Norwich, U.K.) for acquiring a 50-MHz  $^{13}\text{C}$  CPMAS spectrum of a hydrated *Tevnia* tube. S.F.T. and H.C. thank CNRS (France) and The British Council for providing funding for travel between laboratories.

## References and Notes

- Bunn, A.; Cudby, M. E. A.; Harris, R. K.; Packer, K. J.; Say, B. J. *Polymer* 1982, 23, 694.
- Steger, T. R.; Stejskal, E. O.; McKay, R. A.; Stults, B. R.; Schaefer, J. *Tetrahedron Lett.* 1979, 295.
- Belton, P. S.; Tanner, S. F.; Wright, K. M.; Payne, M. P.; Truter, M. R.; Wingfield, J. N. *J. Chem. Soc., Perkin Trans. 2* 1985, 1307.
- Chanzy, H.; Henrissat, B.; Vincendon, M.; Tanner, S. F.; Belton, P. S. *Carbohydr. Res.* 1987, 160, 1.
- Hays, G. R. *J. Chem. Soc., Perkin Trans. 2* 1983, 1049.
- VanderHart, D. L.; Atalla, R. H. *Macromolecules* 1984, 17, 1465.

- (7) Belton, P. S.; Tanner, S. F.; Cartier, N.; Chanzy, H. *Macromolecules* **1989**, *22*, 1615.
- (8) Veregin, R. P.; Fyfe, C. A.; Marchessault, R. H.; Taylor, M. G. *Macromolecules* **1986**, *19*, 1030.
- (9) Rudall, K. M. *Adv. Insect Physiol.* **1963**, *1*, 257.
- (10) Rudall, K. M.; Kenchington, W. *Biol. Rev.* **1973**, *49*, 597.
- (11) Blackwell, J.; Gardner, K. H.; Kolpak, F. J.; Minke, R.; Clafey, W. B. In *Fiber Diffraction Methods*; French, A. D., Gardner, K. H., Eds.; ACS Symposium Series 141; American Chemical Society: Washington, DC, 1980; p 315.
- (12) Blackwell, J. In *Biopolymers*; Walton, A. G., Blackwell, J., Eds.; Academic Press: New York, 1973; p 220.
- (13) Carlström, D. J. *Biophys. Biochem. Acta* **1961**, *51*, 283.
- (14) Minke, R.; Blackwell, J. *J. Mol. Biol.* **1978**, *120*, 669.
- (15) Lotmar, W.; Picken, L. E. R. *Experientia* **1950**, *6*, 58.
- (16) Dweltz, N. E. *Biochem. Biophys. Acta* **1961**, *51*, 283.
- (17) Blackwell, J. *Biopolymers* **1969**, *7*, 281.
- (18) Gardner, K. H.; Blackwell, J. *Biopolymers* **1975**, *14*, 1581.
- (19) Saito, H.; Tabeta, R.; Hirano, S. *Chem. Lett.* **1981**, 1479.
- (20) Schaefer, J.; Kramer, K. J.; Garbow, J. R.; Jacob, G. S.; Stejskal, E. O.; Hopkins, T. L.; Speirs, R. D. *Science* **1987**, *235*, 1200.
- (21) Saito, H.; Tabeta, R.; Ogawa, K. *Macromolecules* **1987**, *20*, 2424.
- (22) Takai, M.; Shimizu, Y.; Hayashi, J.; Uraki, Y.; Tokura, S. In *Chitin and Chitosan*; Skjåk-Braek, G., Anthonsen, T., Sandford, P., Eds.; Elsevier: London, 1989; p 431.
- (23) Peter, M. G.; Kegel, G.; Keller, R. In *Chitin in Nature and Technology*; Muzzarelli, R., Jeuniaux, C., Gooday, G. W., Eds.; Plenum: New York, 1986; p 21.
- (24) Peter, M. G.; Grün, L.; Förster, H. *Angew. Chem., Int. Ed. Engl.* **1984**, *23*, 638.
- (25) Saito, H.; Tabeta, R.; Hirano, S. In *Chitin and Chitosan, Proceedings of the Second International Conference on Chitin and Chitosan*; Hirano, S., Tokura, S., Eds.; The Japanese Society of Chitin and Chitosan, 1982; p 71.
- (26) Vincendon, M.; Roux, J. C.; Chanzy, H.; Tanner, S. F.; Belton, P. S. *Chitin and Chitosan*; Skjåk-Braek, G., Anthonsen, T., Sandford, P., Eds.; Elsevier: London, 1989; p 437.
- (27) McLachlan, J.; McInnes, A. G.; Falk, M. *Can. J. Botany* **1965**, *43*, 707.
- (28) Jones, M. L. *Bull. Biol. Soc. Wash.* **1985**, *6*, 117.
- (29) Earl, W. L.; VanderHart, D. L. *J. Magn. Reson.* **1982**, *48*, 35.
- (30) VanderHart, D. L. *J. Chem. Phys.* **1986**, *84*, 1196.
- (31) A peak at ca. 34 ppm together with some of the shoulders and very small peaks in Figure 1a arise from residual protein in the diatom spines.
- (32) Hexem, J. G.; Frey, M. H.; Opella, S. J. *J. Chem. Phys.* **1982**, *77*, 3847.
- (33) Naito, A.; Ganapathy, S.; McDowell, C. A. *J. Chem. Phys.* **1981**, *74*, 5393.
- (34) Chippendale, A. M.; Mathias, A.; Aujla, R. S.; Harris, R. K.; Packer, K. J.; Say, B. J. *J. Chem. Soc., Perkin Trans. 2* **1983**, 1357.
- (35) Naito, A.; Ganapathy, S.; McDowell, C. A. *J. Magn. Reson.* **1982**, *48*, 367.
- (36) Veregin, R. P.; Fyfe, C. A.; Marchessault, R. H. *Macromolecules* **1987**, *20*, 3007.
- (37) Horii, F.; Hirai, A.; Kitamaru, R. *Bull. Magn. Reson.* **1983**, *5*, 190.
- (38) Gidley, M. J.; Bociek, M. *J. Am. Chem. Soc.* **1988**, *110*, 3820.
- (39) Horii, F.; Hirai, A.; Kitamaru, R. *Polym. Bull.* **1983**, *10*, 357.
- (40) Tanner, S. F.; Ring, S. G.; Whittam, M. A.; Belton, P. S. *Int. J. Biol. Macromol.* **1987**, *9*, 219.
- (41) Rudall, K. M. In *Conformation of Biopolymers*; Ramachandran, G. N., Ed.; Academic Press: New York, 1967; pp 751-65.
- (42) Gaill, F.; Vuong, R.; Chanzy, H., to be submitted for publication.
- (43) Gaill, F.; Hunt, S. *Marine Ecology-Progress Series* **1986**, *34*, 267.
- (44) A broad shoulder of rather low intensity occurring between 170 and 180 ppm may arise from protein.
- (45) Balimann, G. E.; Groombridge, C. J.; Harris, R. K.; Packer, K. J.; Say, B. J.; Tanner, S. F. *Philos. Trans. R. Soc. London* **1981**, *A299*, 643.
- (46) VanderHart, D. L.; Earl, W. L.; Garroway, A. N. *J. Magn. Reson.* **1981**, *44*, 361.
- (47) Torchia, D. A. *J. Magn. Reson.* **1978**, *30*, 613.
- (48) Zugenmaier, P.; Herth, W.; Lieser, G. *Colloid Polym. Sci.* **1981**, *259*, 472.
- (49) Pfeffer, P. E.; Hicks, K. B. *J. Carbohydr. Chem.* **1984**, *3*, 197.
- (50) Fyfe, C. A.; Stephenson, P. J.; Taylor, M. G.; Bluhm, T. L.; Deslandes, Y.; Marchessault, R. H. *Macromolecules* **1984**, *17*, 501.
- (51) Chanzy, H.; Tanner, S. F., unpublished work.

Registry No. Chitin, 1398-61-4.