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High-Resolution Solid-State ^{13}C Nuclear Magnetic Resonance Spectroscopy of Tunicin, an Animal Cellulose

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ABSTRACT: High-resolution solid-state ^{13}C NMR spectra have been obtained from tunicin, a highly crystalline cellulose of animal origin. The spectra of this material contained relatively narrow lines, and peak multiplicities were observed at C1, C4, and C6. Two-component multiplets at C1 and C4 indicate that tunicin consists principally of the Iβ allomorph (as defined by VanderHart and Atalla). The spectrum of tunicin was compared with those recorded from *Valonia ventricosa* and the crystalline regions of cotton.

Introduction

The crystal structure of native cellulose (Cellulose I) has been investigated by using both X-ray and electron diffraction techniques.[1-3] More recently, a number of studies have employed high-resolution solid-state ^{13}C NMR to obtain structural information on cellulose. The appearance of ^{13}C resonances assigned to crystalline or highly ordered regions of native cellulose is variable and depends on sample origin. Peak multiplicities have been observed (particularly at C1 and C4) and three theories have been proposed to account for the spectral variability and the splitting patterns.[4-6] VanderHart and Atalla[4] have suggested that the more ordered component of native cellulose is composed of two allomorphs, Iα and Iβ, and that any native cellulose can be made up of varying amounts of these two forms. The Iα form gives an intense single line at C1 and a doublet at C4, while Iβ gives doublets at both C1 and C4. Thus, the ^{13}C spectrum of an algal cellulose isolated from *Valonia ventricosa* consists of three-component multiplets at C1 and C4 due to the overlap of resonances arising from the two allomorphs. NMR experiments isolating the more crystalline components of cotton suggest that the native cellulose occurring in higher plants is principally in the Iβ form. Further, the C4 line shape of the Iβ allomorph was probably more complicated than had originally been proposed.[7] Cael et al.[5] have proposed a different model for the interpretation of native cellulose spectra which is based on diffraction data. They suggest that cellulose consists of varying amounts of material characterized by either eight or two chain unit cells. The two-chain cell gives a two-component 1:1 multiplet, while the eight-chain cell gives a "triplet" with an intensity ratio of 1:2:1. *V. ventricosa* contains only eight-chain unit cell material and, according to the model, gives a three-component multiplet at both C1 and C4. The intense central component arises from four similar chains labeled A, B, C, and D, and the surrounding weaker peaks are assigned to two chains labeled E and F.

In this report, we present the high-resolution ^{13}C spectrum of a native cellulose isolated from pelagic tunicates. This spectrum is the first to be obtained from cellulose of animal origin. The tunicate cellulose (hereafter referred to as tunicin) is of special interest because its preparation requires only mild chemical and enzymic treatment. This is in marked contrast to the severe treatment carried out on low DP regenerated cellulose,[4] a material giving a similar spectrum to tunicin. Examination of tunicin using an electron microscope shows that it is highly crystalline[8] and consists of randomly intertwined cellulose microfibrils.[9] These microfibrils are approximately 10 nm in width, and like *V. ventricosa*, each tunicin microfibril is a distinct cellulose crystal having the full width of the microfibril.[10] Tunicin cellulose is less crystalline than *V. ventricosa* but

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Table I
^{13}C NMR Chemical Shifts of Cellulose Samples

sample	C-1	C-4	C2,C3,C5		C6
Valonia	106.2	90.4	75.3	72.2	65.8
ventricosa	105.6	89.4	74.7	71.8	65.5
	104.5	88.6	73.1	71.4	
tunicin	106.2		75.4		66.0
		89.3	74.7	71.8	65.4
	104.5	88.5	72.8		

is more crystalline than bacterial or cotton cellulose. This is reflected in the low susceptibility of tunicin to mercerization.[8] In *V. ventricosa*, the cellulose microfibrils have a lateral size and crystallinity greater than in tunicin and are highly organized, being packed in parallel within wall layers that are stacked in a criss-crossed fashion. In a typical mature *V. ventricosa* cell wall, there may be up to 50 of these layers.

Experimental Section

A crop of the pelagic tunicates (*Salpa fusiformis*) was harvested in the Bay of Villefranche near Nice, France. The tunicates, preserved in ethanol, were slit open with the aid of a stereomicroscope, and most of the non-cellulosic components were removed by using tweezers. The cellulosic remainder (the tunicin) and the attached muscle were digested using pronase (from Boehringer Mannheim GmbH) for 3 days at 40 °C in a phosphate buffer at pH 7.5. 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 using 1% NaOH under a nitrogen atmosphere for 4 h. After neutralization and washing, the tunicin was stored in ethanol until required. It was found to have a carbohydrate content consisting of more than 99% glucose. *Valonia ventricosa* cell wall fragments were prepared according to the method described by Gardner and Blackwell.[1]

Results and Discussion

The high-resolution solid-state ^{13}C spectrum of tunicin is shown in Figure 1, together with the spectrum of *V. ventricosa*. Also shown are resolution-enhanced spectra of the same materials. Chemical shifts and assignments (where possible) are displayed in Table I. The ^{13}C spectrum of tunicin contains relatively narrow lines, consistent with a high degree of crystallinity. A number of carbon atoms that give rise to narrow lines in the tunicin spectrum show peak multiplicities. Thus C1, C4, and C6 consist of imperfectly resolved multiplets containing two major components. The remaining carbon atoms (C2, C3, and C5) have not been assigned, and consequently it is difficult to be sure of splittings occurring in this region of the spectrum (~70–80 ppm).

Comparison of ^{13}C spectra and chemical shifts reveals that, within experimental error, the tunicin spectrum is a subset of the *V. ventricosa* spectrum. *V. ventricosa* contains all the peaks found in tunicin in addition to some extra resonances. Thus, at C1, tunicin displays an approximately 1:1 multiplet with chemical shifts identical with the outer lines of the three-component *V. ventricosa* multiplet (see Table I). The spectrum of tunicin is remarkable in that it is identical with the cellulose Iβ spectrum originally simulated by VanderHart and Atalla.[4] The Cael et al. model[5] would necessarily characterize tunicin using a two-chain unit cell, as the line shapes at C1 and C4 consist of only two principal components. However, this model is inconsistent in the detailed assignments of the C1 and C4 tunicin resonances. At C1, the two peaks have chemical shifts characterized by the model as arising from only E- and F-type chains, while at C4 A-, B-, C-, D-, E-, or F-type resonances are detected.

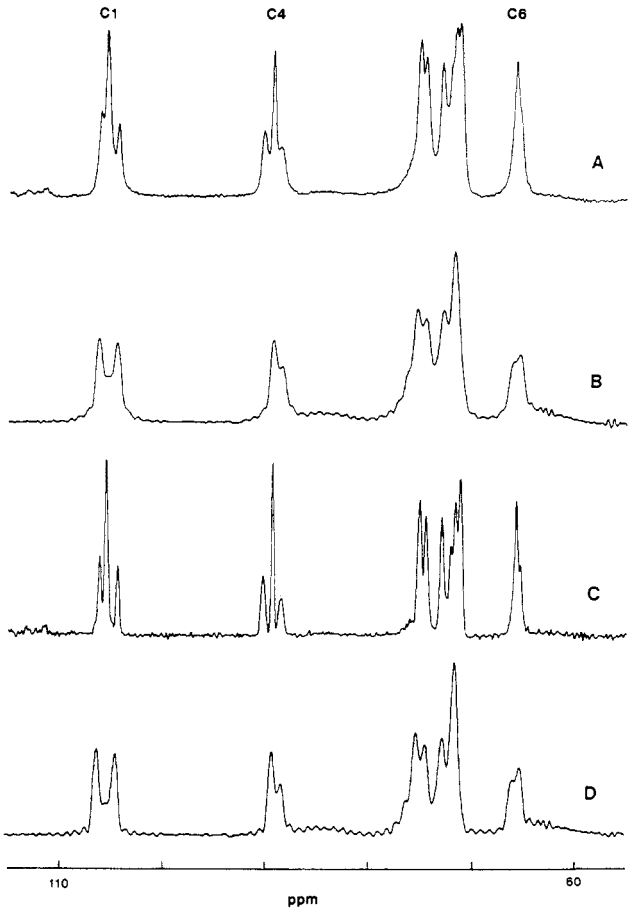

Figure 1. ^{13}C solid-state NMR spectra acquired at 75 MHz of (A) *Valonia ventricosa* and (B) tunicin. Spectra C and D have been resolution enhanced by multiplication of the free-induction decay using a function of the form $\exp(-at - bt^2)$, where $a = \pi LB$, $b = -a/(2(GB)(AQ))$, LB = −30 Hz, GB = Gaussian broadening factor = 0.5, and AQ = acquisition time = 57 ms.

This combination cannot arise either from eight- or two-chain unit cells or from a combination of both of these materials.

^{13}C spectra of cotton and tunicin are shown in Figure 2. These spectra were acquired by using a pulse program developed by Torchia[11] to measure ^{13}C spin–lattice relaxation times (^{13}C T_1). Crystalline regions within native cellulose samples are assumed to have longer ^{13}C T_1 values than the amorphous domains. The application of the T_1 pulse program discriminates in favor of carbon nuclei that possess a long T_1, that is, in favor of crystalline components within the sample. Close examination of Figure 2 reveals that, although the spectra are generally similar, some differences exist between tunicin and the crystalline components of cotton. Cotton shows a distinct high-frequency shoulder at C4 (marked with an asterisk), which is either absent or extremely weak in the tunicin spectrum. (This shoulder is present in the cotton spectrum even if very long delays are used in the T_1 pulse program.[7]) Recently it has been suggested that the crystalline components of cotton represent material which is principally in the cellulose Iβ form. We believe the tunicin spectrum is more representative of the Iβ allomorph. The spectrum of tunicin is simpler and is more highly resolved than the corresponding cotton spectrum. Further, as has been stated, it agrees remarkably well with that originally proposed for Iβ cellulose.[4]

The NMR lines at C1 and C4 have proved difficult to interpret in spectra of native cellulose. The C1 region is particularly complex in that it consists of a superposition

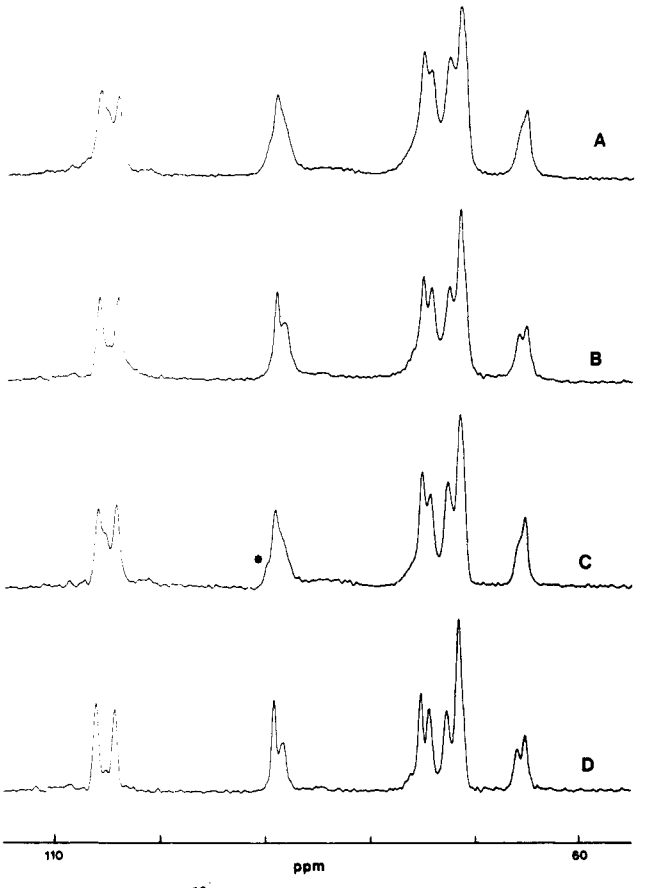

Figure 2. 75-MHz ^{13}C solid-state NMR spectra of (A) cotton and (B) tunicin using the T_1 pulse sequence (ref 11) and a τ value of 30 s. Spectra C and D are of cotton and tunicin, respectively, acquired under identical conditions and resolution enhanced (as in Figure 1).

of signals arising from both crystalline and amorphous material. This complication is not thought to occur at C4 since resonances arising from crystalline and amorphous domains are well separated. Interpretation of the C4 region is still difficult, however. The *V. ventricosa* spectrum consists of a three-component multiplet in this region. The two outer components are broader than the middle line which occurs at ~89.4 ppm. Tunicin is somewhat similar in that the low-frequency member of the C4 multiplet is also broader than the line at ~89.3 ppm. It has been suggested that differences between the two proposed allomorphs of native cellulose arise through differences in hydrogen-bonding patterns.[12] If this is true, then it is similar to observations made on some α(1–4)-linked glucans in which the C4 resonances are also found to be highly sensitive to hydrogen bonding.[13] Recent work on two crown ether complexes which have well-characterized crystal structures and apparently identical carbon skeletons shows differences in their ^{13}C spectra that cannot be easily interpreted.[14] Differences between *V. ventricosa*, tunicin, and cotton spectra may therefore reflect only tiny differences in the local environments of the crystalline regions within these samples. There are, however, differences in the ultrastructure and in the high-resolution spectra of *V. ventricosa* and tunicin. We suggest, therefore, that a detailed comparison of ultrastructure and its relationship to differences in local molecular environments is required in order to understand the differences in the NMR spectra of native cellulose samples.

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