# NUCLEAR MAGNETIC RESONANCE ANALYSIS OF WATER IN NATURAL AND DEUTERATED MOUSE MUSCLE ABOVE AND BELOW FREEZING

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ABSTRACT Measurments of absolute proton signal intensities, free induction decays, spin-spin relaxation times, and local fields in the rotating frame in natural and fully deuterated mouse muscle at five temperatures in the range 293–170 K are reported. The analysis is carried out at three time windows on the free induction decay. The contribution to the magnetization from protons on large molecules and water are analyzed.

## INTRODUCTION

To increase the applicability of the nuclear magnetic resonance (NMR) spectroscopy in biological systems it is necessary to enhance the resolution of the distribution of spins with different degrees of mobility and/or different anisotropy of their motion. Numerous NMR studies were performed to further the understanding about these spin distributions (1-23). Several NMR experiments (9, 14, 20, 23) indicate that in muscle tissue the magnetization distribution consists of five distinct components: a solid (S), semisolid (SS), semiliquid (SL) and two liquid (L) components. In general, the large molecule (LM) and water proton spin-spin relaxation time $T_2$ of a particular component are so similar that the separation of LM and $H_2O$ contribution to this component on the basis of $T_2$ is not possible in the natural sample. The contributions to the total magnetization may be determined by comparing the absolute signal intensities in natural (n-) and deuterated (d-) muscle samples. In this study, the magnetization components were resolved and their local fields determined. The composition of the proton magnetization distribution in terms of LM protons and $H_2O$ protons is given in Table I in the "liquid like" phase I (293 and 269 K), the "waxy" phase II (256 and 238 K), and the "glassy" phase III (170 K), (see reference 22). All results are obtained at the 16- and 200-$\mu$s time windows on the free induction decay (FID). Also, the changes of proton magnetization distribution brought about by tissue freezing are reported and water solidification is briefly discussed.

## METHODS

Spin echo envelopes and FID were measured with a SXP Bruker pulse spectrometer (Bruker Instruments, Inc. Billerca, Mass.) at 33.8 MHz. Since the receiver dead time was ~6 $\mu$s, the FID's were recorded with an 8-$\mu$s delay (after the 90° pulse). $T_2$ was measured using the Gill-Meiboom modified Carr-Purcell pulse sequence with $2\tau = 60$ $\mu$s. A CP-2 Spin-Lock spectrometer (Spin-Lock Ltd., Ontario, Canada) operating at 33.8 MHz was used for $H_1'$ measurements. In all experiments the signal averaging

was performed utilizing a Fabritek 1072 (Fabritek Co., Inc., Winchester, Va.) in conjunction with a Biomation 805 sample and hold unit (Biomation, Cupertino, Calif.). The averaging varied typically from 32 accumulations in n-muscle at 293 K to 2,048 accumulations in d-muscle at 238 K.

All experiments were preformed on muscle tissue of 4-wk-old C57 black mice. The tissue samples were blotted free of blood, cut into ~0.1-$cm^3$ pieces, placed in a 2-cm long glass tube of 0.5 cm i.d. and sealed. The osmotically balanced (isotonic) deuteration was achieved by immersion in a phosphate-buffered saline (PBS) 99.8% $D_2O$ solution for two periods of 4 hr each; the first immersion was carried out at room temperature and the second in fresh PBS solution at 5°C. The samples were obtained from Dr. W. R. Inch, The Ontario Cancer Treatment and Research Foundation, London Clinic, London, Ontario, Canada.

The temperature dependence of the probe circuit was monitored by measuring the signal intensity at a 16-$\mu$s window of a $CH_2Cl_2$ sample of approximately the same volume as that of the muscle samples. Within the temperature range 293–170 K, the signal depended on temperature as expected (Curie law). All muscle signal intensities recorded with the same spectrometer setting at different temperatures (Table I) were normalized with respect to the signal intensity of n-muscle at room temperature. The above normalizations had to be carried out on signal intensities projected to zero time since the solid-like components of the magnetization in n- and d-muscle have slightly different $T_2$ (Table I). Such normalized NMR signals are measures of the number of all proton spins in a given sample.

Since freezing affects the muscle tissue irreversibly, each sample was used for only one cooling run. For this reason n-muscle and d-muscle temperature dependencies had to be studied on different tissues. This necessitates the normalization of the d-muscle signal intensity to that of the n-muscle intensity, which was accomplished by comparing the dry weights of d- and n-muscle. The muscles were dried in a vacuum for 36 h; over the last 24 h the pressure was $\sim 10^{-3}$ Torr. To monitor the drying process, the samples were weighed after 36 h of drying and were returned to the vacuum for an additional 36 h. Upon reweighing, no change in weight was observed.

## RESULTS

The results of FID $T_2$, $H_\ell'$ and normalized signal intensities of n- and d-muscle in phase I at 293 and 269 K, in phase II at 256 and 238 K, and in phase III at 170 K are shown in Table I. The FID, $T_2$ and $H_\ell'$ results at 293, 269, and 256 K had been reported earlier (23), but the magnetizations were not normalized. As a result, no quantitative comparative analysis of liquid-like and solid-like protons was attempted. Such analysis is reported below.

To relate the LM magnetization in the d-muscle to that of the LM magnetization in n-muscle a normalization factor (signal intensity of d-muscle vs. signal intensity of n-muscle) of 0.245 ± 0.048 was derived. This factor is an average over a set of signal intensities and dry weights in six n-muscle and in six d-muscle samples. Since all magnetization fractions have been normalized with respect to the n-muscle at 293 K and at the 0-$\mu$s window (Table I), it is possible to read the effect of temperature on magnetization intensities in n- and d-muscle directly from Table I (horizontal analysis). Furthermore, it is possible to determine, with some loss of accuracy, the contributions to a specific magnetization component from LM protons and from $H_2O$ protons by subtracting the d-muscle magnetization from that of n-muscle magnetization (see Table I, vertical analysis).

## DISCUSSION

At 293 K, the magnetization decay curves in n- and d-muscle are resolved into four components each (see Table I). The component in n-muscle with $T_2 = (41 \pm 1)$ ms is not observed in d-muscle and hence is due to water. It has been noted (23) that the L-magnetization

TABLE IA

MAGNETIZATIONS DERIVED FROM FID AND $T_2$ RESULTS IN NATURAL AND FULLY DEUTERATED MUSCLE TISSUE

Natural tissue (LM + $H_2O$ magnetization

| Window | 293 K | | | | |
|---|---|---|---|---|---|
| | Magnetization | | | $H'_f$ | $T_2$ |
| | 0 | 16 | 200 | | |
| L | 6.8 ± 0.8 | 6.8 ± 0.8 | 6.8 ± 0.8 | 0 | 143 ± 6 |
| | 76.1 ± 2.1 | 76.0 ± 2.1 | 75.7 ± 2.1 | | 41 ± 1 |
| SL | 6.3 ± 0.4 | 6.3 ± 0.4 | 6.1 ± 0.4 | 0 | 5.3 ± 0.4 |
| SS | | | | | |
| S | 10.8 ± 0.3 | 7.9 ± 0.3 | 0 | 1.0 ± 0.1 | ~24 × $10^{-3}$ |
| Total | 100.0 ± 2.3 | 97.0 ± 2.3 | 88.6 ± 2.3 | | |
| | 269 K | | | | |
| Window | Magnetization | | | $H'_f$ | $T_2$ |
| | 0 | 16 | 200 | | |
| L | 5.2 ± 1.4 | 5.2 ± 1.4 | 5.1 ± 1.4 | 0 | 148 ± 12 |
| | 75.8 ± 1.6 | 75.8 ± 1.6 | 74.7 ± 1.6 | | 41 ± 1 |
| SL | 5.9 ± 0.4 | 5.9 ± 0.4 | 5.7 ± 0.4 | 0 | 7.7 ± 0.5 |
| SS | | | | | |
| S | 14.0 ± 0.9 | 8.2 ± 0.6 | 0 | 0.9 ± 0.2 | ~18 × $10^{-3}$ |
| Total | 100.9 ± 2.3 | 95.1 ± 2.2 | 85.5 ± 2.2 | | |
| | 256 K | | | | |
| Window | Magnetization | | | $H'_f$ | $T_2$ |
| | 0 | 16 | 200 | | |
| L | 0 | 0 | 0 | | |
| SL | 12.8 ± 0.6 | 12.7 ± 0.6 | 12.3 ± 0.6 | 0 | 5.2 ± 0.1 |
| SS | 1.2 ± 0.5 | 1.2 ± 0.5 | 0.7 ± 0.5 | 0.5 ± 0.2* | 0.35 ± 0.04 |
| S | 16.0 ± 1.2 | 8.0 ± 0.6 | 0 | 1.7 ± 0.3* | ~16 × $10^{-3}$ |
| Total | 30.0 ± 1.4 | 21.9 ± 1.0 | 13.0 ± 0.8 | | |
| | 238 K | | | | |
| Window | Magnetization | | | $H'_f$ | $T_2$ |
| | 0 | 16 | 200 | | |
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 10.5 ± 1.2 | 10.2 ± 1.2 | 7.2 ± 0.6 | 0.3 ± 0.1* | 0.53 ± 0.01 |
| S | 19.1 ± 2.4 | 6.9 ± 1.1 | 0 | 1.8 ± 0.3* | ~14 × $10^{-3}$ |
| Total | 29.6 ± 2.8 | 17.1 ± 1.6 | 7.2 ± 0.6 | | |
| | 170 K | | | | |
| Window | Magnetization | | | $H'_f$ | $T_2$ |
| | 0 | 16 | 200 | | |
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 0 | 0 | 0 | | |
| S | 29.4 ± 1.9 | 5.0 ± 0.4 | 0 | 1.0 ± 0.3*<br>2.9 ± 0.4* | ~10 × $10^{-3}$ |
| Total | 29.4 ± 1.9 | 5.0 ± 0.4 | 0 | | |

All magnetizations are normalized with respect to natural tissue at the temperature and window of 293 K and 0 μs, respectively. $H'_f$, $T_2$, and the time windows are given in G, ms, and μs, respectively. A small percentage of the $H_2O$ magnetization is due to exchangeable NH and OH protons (23). We estimate that this percentage is at least 1.6% but not greater than 2.6% of the total magnetization at 293 K.

*Estimates.

TABLE IB

MAGNETIZATIONS DERIVED FROM FID AND $T_2$ RESULTS IN NATURAL AND FULLY DEUTERATED MUSCLE TISSUE

Fully deuterated tissue (LM) magnetization

| Window | 293 K Magnetization 0 | 16 | 200 | $H'_\ell$ | $T_2$ |
|---|---|---|---|---|---|
| L | 8.7 ± 1.7 | 8.7 ± 1.7 | 8.7 ± 1.7 | 0 | 122 ± 2 |
| SL | 1.8 ± 0.4 | 1.8 ± 0.4 | 1.8 ± 0.4 | 0 | 9.8 ± 0.5 |
| SS | 3.4 ± 0.7 | 3.3 ± 0.7 | 2.2 ± 0.4 | 0.5 ± 0.2 | 0.44 ± 0.04 |
| S | 10.6 ± 2.1 | 6.3 ± 1.2 | 0 | 0.9 ± 0.1 | $\sim 18 \times 10^{-3}$ |
| Total | 24.5 ± 2.8 | 20.1 ± 2.2 | 12.7 ± 1.8 | | |

| Window | 269 K Magnetization 0 | 16 | 200 | $H'_\ell$ | $T_2$ |
|---|---|---|---|---|---|
| L | 7.7 ± 1.5 | 7.7 ± 1.5 | 7.7 ± 1.5 | 0 | 127 ± 2 |
| SL | 2.6 ± 0.5 | 2.6 ± 0.5 | 2.6 ± 0.5 | 0 | 15.0 ± 1.2 |
| SS | 2.5 ± 0.5 | 2.4 ± 0.5 | 2.0 ± 0.4 | 0.3 ± 0.1 | 0.98 ± 0.09 |
| S | 12.2 ± 2.4 | 6.8 ± 1.4 | 0 | 1.3 ± 0.1 | $\sim 18 \times 10^{-3}$ |
| Total | 25.0 ± 2.9 | 19.5 ± 2.2 | 12.3 ± 1.6 | | |

| Window | 256 K Magnetization 0 | 16 | 200 | $H'_\ell$ | $T_2$ |
|---|---|---|---|---|---|
| L | 0 | 0 | 0 | | |
| SL | 3.0 ± 0.6 | 3.0 ± 0.6 | 2.9 ± 0.6 | 0 | 7.5 ± 1.2 |
| SS | 1.4 ± 0.4 | 1.4 ± 0.4 | 0.9 ± 0.3 | 0.5 ± 0.2* | 0.43 ± 0.04 |
| S | 19.7 ± 3.9 | 8.1 ± 1.6 | 0 | 1.5 ± 0.3* | $\sim 11 \times 10^{-3}$ |
| Total | 24.1 ± 4.0 | 12.5 ± 1.8 | 3.8 ± 0.8 | | |

| Window | 238 K Magnetization 0 | 16 | 200 | $H'_\ell$ | $T_2$ |
|---|---|---|---|---|---|
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 2.4 ± 0.5 | 2.3 ± 0.5 | 1.8 ± 0.4 | 0.3 ± 0.1* | 0.67 ± 0.05 |
| S | 21.9 ± 4.4 | 8.4 ± 1.7 | 0 | 1.6 ± 0.2* | $\sim 11 \times 10^{-3}$ |
| Total | 24.3 ± 4.4 | 10.7 ± 1.8 | 1.8 ± 0.4 | | |

| Window | 170 K Magnetization 0 | 16 | 200 | $H'_\ell$ | $T_2$ |
|---|---|---|---|---|---|
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 0 | 0 | 0 | | |
| S | 24.7 ± 4.9 | 7.5 ± 1.5 | 0 | 0.8 ± 0.2*<br>2.5 ± 0.4* | $\sim 10 \times 10^{-3}$ |
| Total | 24.7 ± 4.9 | 7.5 ± 1.5 | | | |

All magnetizations are normalized with respect to natural tissue at the temperature and window of 293 K and 0 $\mu$s, respectively. $H'_\ell$, $T_2$, and the time windows are given in G, ms, and $\mu$s, respectively. A small percentage of the $H_2O$ magnetization is due to exchangeable NH and OH protons (23). We estimate that this percentage is at least 1.6% but not greater than 2.6% of the total magnetization at 293 K.

*Estimates.

TABLE IC

MAGNETIZATIONS DERIVED FROM FID AND $T_2$ RESULTS IN NATURAL AND FULLY DEUTERATED MUSCLE TISSUE

$H_2O$ Magnetization

| Window | 293 K Magnetization 0 | 16 | 200 | $H_g'$ | $T_2$ |
|---|---|---|---|---|---|
| L | 74.2 ± 2.8 | 74.1 ± 2.8 | 73.8 ± 2.8 | 0 | ~40 |
| SL | 4.5 ± 0.6 | 4.5 ± 0.6 | 4.3 ± 0.6 | 0 | ~8 |
| SS | −3.4 ± 0.7 | −3.3 ± 0.7 | −2.2 ± 0.4 | | |
| S | −0.2 ± 2.1 | 1.6 ± 1.2 | 0 | | |
| | **269 K** | | | | |
| Window | Magnetization 0 | 16 | 200 | $H_g'$ | $T_2$ |
| L | 73.3 ± 2.6 | 73.3 ± 2.6 | 2.1 ± 2.6 | 0 | ~40 |
| SL | 3.3 ± 0.6 | 73.3 ± 0.6 | 3.1 ± 0.6 | 0 | ~8 |
| SS | −2.5 ± 0.5 | −2.4 ± 0.5 | −2.0 ± 0.4 | | |
| S | 1.8 ± 2.6 | 1.4 ± 1.5 | 0 | | |
| | **256 K** | | | | |
| Window | Magnetization 0 | 16 | 200 | $H_g'$ | $T_2$ |
| L | 0 | 0 | 0 | | |
| SL | 9.8 ± 0.8 | 9.7 ± 0.8 | 9.4 ± 0.8 | 0 | ~5 |
| SS | −0.2 ± 0.6 | −0.2 ± 0.6 | −0.2 ± 0.6 | | |
| S | −3.7 ± 4.2 | −0.1 ± 2.1 | 0 | | |
| | **238 K** | | | | |
| Window | Magnetization 0 | 16 | 200 | $H_g'$ | $T_2$ |
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 8.1 ± 1.3 | 7.9 ± 1.9 | 5.4 ± 0.7 | | |
| S | −2.8 ± 5.0 | −1.5 ± 2.0 | 0 | | |
| | **170 K** | | | | |
| Window | Magnetization 0 | 16 | 200 | $H_g'$ | $T_2$ |
| L | 0 | 0 | 0 | | |
| SL | 0 | 0 | 0 | | |
| SS | 0 | 0 | 0 | | |
| S | 4.7 ± 5.3 | −2.5 ± 1.6 | 0 | | ~$10 \times 10^{-3}$ |

All magnetizations are normalized with respect to natural tissue at the temperature and window of 293 K and 0 μs, respectively. $H_g'$, $T_2$, and the time windows are given in G, ms, and μs, respectively. A small percentage of the $H_2O$ magnetization is due to exchangeable NH and OH protons (23). We estimate that this percentage is at least 1.6% but not greater than 2.6% of the total magnetization at 293 K.

*Estimates.

fraction in n-muscle with $T_2$ = (143 ± 6) ms could be due to either the LM or water protons. This fraction has a $T_2$ similar to that of the L-component in d-muscle (122 ± 2 ms). We conclude that the 143-ms fraction is due to the LM protons since its magnitude (6.8 ± 0.8)% and its $T_2$ are similar to the magnitude (8.7 ± 1.7)% and $T_2$ in d-muscle.

The possibility that the L-component in d-muscle, $T_2$ = (122 ± 2) ms, is due to trapped $H_2O$ molecules which did not exchange during the deuteration was investigated as follows: A deuterated muscle sample was dried at room temperature in a vacuum for 72 h and the $D_2O$ evaporated from this sample was collected in a cold trap. No proton NMR signal could be detected from this $D_2O$ sample using the same spectrometer settings as were used for the other experiments. Thus, the muscle water has been replaced totally by $D_2O$ during deuteration.

At 293 K, the SS-component of (3.4 ± 0.7)% was observed in d-muscle but not in n-muscle. This component was resolved in d-muscle since it amounts to 14% of the total magnetization in d-muscle (but only to 3.4% of the total magnetization in n-muscle). It may be noted that in the present experiment five magnetization fractions in muscle tissue were resolved, each with a different $T_2$ value.

Comparing magnetizations of n- and d-muscles (the vertical analysis) at 293 K shows that (74.2 ± 2.8)% and (4.5 ± 0.6)% of the magnetization in n-muscle is due to L-like and SL-like $H_2O$ protons, respectively. These two fractions, arrived at by subtracting the d-muscle value from the n-muscle value, are shown in the bottom row of Table I as $H_2O$ magnetization. The negative $H_2O$ magnetization fractions (appearing when the d-muscle magnetization is subtracted from the n-muscle magnetization) are the result of two factors. In the first place, the measurements in n- and d-muscle do not resolve a particular component equally. This is illustrated by the previous discussion on the SS-component with $T_2$ = (0.44 ± 0.4) ms, which was resolved in d-muscle only. In addition, the actual value of a particular magnetization fraction varies from sample to sample by as much as ±10% of its average value.

In n- and d-muscle, at 293 K, the S-components are (10.8 ± 0.3)% and (10.6 ± 2.1)%, respectively (Table I). This could only mean that the S-component in n-muscle is due solely to LM protons. As a result, the S-component of the $H_2O$ magnetization (Table I) is equal to zero within experimental uncertainty.

The horizontal analysis in n- and d-muscle in phase I shows that in this phase the results are essentially temperature independent. The exception is the S-component in n-muscle, which increases from (10.8 ± 0.3)% to (14.0 ± 0.9)% as the temperature is lowered from 293 to 269 K (Table I). This demonstrates that a minor slowing down, or increased anisotropy, of molecular motion occurs already at −4°C.

Upon freezing a large redistribution of relative fractions is observed. It occurs that the magnetization fractions (normalized to the magnetization at 293 K in n-muscle at the 0-$\mu$s window) either disappear upon freezing the tissue or remain the same within the accuracy of the experiment. For example, the L-component in n- and d-muscle disappears upon freezing the tissue (Table I). In n-muscle this is the result of the freezing of most of the $H_2O$ (70% of the magnetization). In d-muscle most of the $D_2O$ also freezes, and as a result the mobility of some parts of LM is reduced. Thus, most of the protons on these solidified parts of LM now undergo sufficiently slow and/or sufficiently anisotropic motion to appear as S-protons (Table I).

The SS-component which was not resolved in n-muscle in phase I appears at 256 K as a

(1.2 ± 0.5)% fraction, which is ~4% of the remaining magnetization, with $T_2$ = (0.35 ± 0.04) ms. A similar component is found in d-muscle, (1.4 ± 0.4)% with $T_2$ = (0.43 ± 0.04) ms. These values agree within experimental uncertainty, supporting the above suggestion that the SS-component in n-muscle is due to LM protons.

At 256 K the total magnetization in n-muscle and d-muscle is (30.0 ± 1.4)% and (24.1 ± 4.0)%, respectively. Consequently, only 30.0–24.1 = (5.9 ± 4.2)% of the magnetization in n-muscle at 256 K is due to protons on $H_2O$ molecules. This large uncertainty (±4.2%) shows the limitations of the "vertical analysis" mentioned above. It is interesting to note, that at the 200-$\mu$s window the LM protons contribute only a few percent while water contributes the rest.

$T_2$ of the S-component in n-muscle does not change much (from 18 to 16 $\mu$s) as the sample is frozen. The corresponding change in $H_\ell'$ is larger, from (0.9 ± 0.2) to (1.7 ± 0.3) G, respectively. A more visible change in the corresponding $T_2$ appears in d-muscle. The $T_2$ ~18 $\mu$s at 269 K decreases to ~11 $\mu$s at 256 K. This decrease in $T_2$ in d-muscle is accompanied by an increase in $H_\ell'$ from (1.3 ± 0.1) G at 269 K to (1.5 ± 0.3) G at 256 K.

At 238 K, the SL-component is not observed in n- and d-muscle (Table I). Since in each case the total magnetization observed remains unchanged, it follows that the increase in the SS- and S-component at 238 K is contributed by the protons which formed the SL-component at 256 K. On cooling from 256 to 238 K, the $T_2$ of the SS-component increases from (0.35 ± 0.04) ms to (0.53 ± 0.01) ms in n-muscle, and from (0.43 ± 0.04) ms to (0.67 ± 0.05) ms in d-muscle. The apparent unusual behavior is the result of a magnetization component mixing. At 238 K, a major fraction of the SS-magnetization is contributed by the protons which formed the SL-component at 256 K. At 238 K, these protons are more mobile and/or their motion is more isotropic than the protons which were classified SS-protons at 256 K.

The vertical analysis at 238 K shows that $H_2O$ contributes ~5.3% to the total magnetization of 29.6% in n-muscle (Table I). At this temperature, the magnetization in n-muscle at the 200-$\mu$s window is mostly due to $H_2O$ which contributes ~5.4% to the magnetization of 7.2% (Table I).

At 170 K in n- and d-muscle, only the S-component remains observable (Table I). In each case the total magnetization is equal to the sum of the magnetization fractions at 238 K within experimental uncertainty. Thus, the SS-components at 238 K become part of the S-component at 170 K. The sum of all the fractions at 170 K are, in n-muscle, (29.4 ± 1.9)%, and in d-muscle, (24.7 ± 4.0)%. From this experiment it follows that the $H_2O$ magnetization at 170 K is 4.7 ± 5.3%. Because of this low accuracy at 170 K, no conclusion can be drawn about the state of water from the vertical analysis. However, with the horizontal analysis in phase II and III in n-muscle the water has been identified with better accuracy as follows: The total n-muscle magnetization and the total d-muscle magnetization remain constant from 256 to 170 K. The experimental uncertainty for n-muscle magnetization magnitude is ±2.8% or better, through phase II and into phase III (Table I). Thus, the same number of $H_2O$ protons is observed at 170 K as was observed at 256 K, since neither the LM magnetization nor the total magnetization had changed appreciably. Even at 170 K, some of the $H_2O$ has not frozen (although it had solidified). This observation is in qualitative accord with calorimetric measurements (24) of the specific heat in collagen with different contents of water, where it was observed that some $H_2O$ had not frozen even at temperatures below 50 K. Despite the

large uncertainties quoted with the vertical analysis, the $H_2O$ percentage of 4.7% at 170 K (Table I) is more accurate than shown because of the correlations between values at 256, 238, and 170 K.

It is possible to conclude, therefore, that all freezing of $H_2O$ molecules occurs at the freezing phase transition (~265 K). Any solidifying of the remaining $H_2O$ leads to a polymer-$H_2O$ solid different from ice. This solid has $T_1$ some three orders of magnitude shorter than ice. At 170 K, its $T_2$ is of the order 10 $\mu$s.

In summary, the proton magnetization distribution in n-muscle has been resolved into LM and $H_2O$ contributions by a comparative analysis of normalized signal intensities in n- and d-muscle (Table I). Five magnetization components were resolved from the FID and $T_2$ in muscle at 293 K. The vertical analysis shows that the L-component (with $T_2$ = 143 ms) and the S-component (with $T_2$ = ~24 $\mu$s) are due to LM protons as well. The L-component (with $T_2$ = ~40 ms) is due to water only. The SL-component, (with $T_2$ ~5 ms) is due to water (~70%) and LM protons (~30%). Considering only the SL-component at 256 K, in n-muscle, under the condition of saturated hexagonal ice magnetization, 9.8% of the proton magnetization at 293 K is nonfreezable (SL) $H_2O$ proton magnetization. Freezing of the majority of $H_2O$ occurs only at the freezing phase transition.

It is also shown that the assumptions about a particular magnetization component being due to $H_2O$ or LM protons must be taken with care. For example, in the literature it is commonly assumed that the L-components in n-muscle in phase I are due to $H_2O$ protons only. In the present work it is shown that this does not hold (Table I).

More information on the magnetization distribution and an improvement in the resolution into magnetization components can be achieved by increasing the number of time windows. This approach is presently being pursued with an on-line computer.

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