STUDY OF ANISOTROPY IN NUCLEAR MAGNETIC RESONANCE RELAXATION TIMES OF WATER PROTONS IN SKELETAL MUSCLE

S. R. KASTURI, *Tata Institute of Fundamental Research, Colaba, Bombay, 400005, India*

D. C. CHANG AND C. F. HAZLEWOOD, *Departments of Pediatrics and Physiology, Baylor College of Medicine, Houston, Texas 77030, and Department of Physics, Rice University, Houston, Texas 77005 U.S.A.*

ABSTRACT The anisotropy of the spin-lattice relaxation time (T_1) and the spin-spin relaxation times (T_2) of water protons in skeletal muscle tissue have been studied by the spin-echo technique. Both T_1 and T_2 have been measured for the water protons of the tibialis anterior muscle of mature male rats for $\theta = 0$, 55, and 90°, where θ is the orientation of the muscle fiber with respect to the static field. The anisotropy in T_1 and T_2 has been measured at temperatures of 28, −5 and −10°C. No significant anisotropy was observed in the T_1 of the tissue water, while an average anisotropy of ~5% was observed in T_2 at room temperature. The average anisotropy of T_2 at −5 and −10°C was found to be ~2 and 1.3%, respectively.

INTRODUCTION

Water and ions are major constituents of a living cell. In recent years, there has been considerable interest in the study of the physico-chemical state of these constituents using nuclear magnetic resonance (NMR) (1–28) and other physico-chemical techniques (1,29, 30–43).

The concept of "ordering" of water has been suggested in some of these studies (4,10,11,15,24,42), and the hypothesis has been advanced that interactions between the water molecules and the biopolymer interface result in a reduction in the mobility of the water molecules in the cell. This hypothesis implies that the correlation times (rotational and/or translational) for these water molecules are longer in tissues than those of bulk water. NMR experiments show that partial orientation (or ordering) of the water molecules due to these interactions exists in hydrated collagen (44–48), oriented DNA (49), oriented rayon fibers (50), frog muscle tissue (51), and other systems (52). In nerve tissue, however, there is some controversy regarding the existence of oriented water (52,53).

Testing for the presence of oriented water, Finch et al. (9) studied the water in resting muscle and observed no anisotropic effect on either T_2 or the diffusion coefficient of cellular water.[1] In contrast, Cleveland et al. (24) observed a significant anisotropy (28%) in the

[1]In a personal communication to Dr. Hazlewood, Finch indicated that his attempts to measure anisotropy were hampered by the fact that he could not be certain that he could properly align small pieces of muscle in the NMR sample tubes.

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diffusion coefficients of intracellular water in skeletal muscle measured with the muscle fiber orientation parallel and perpendicular to the direction of diffusion.

In our earlier studies, we observed an anisotropy in the spin-spin relaxation time of water in rat skeletal muscle.[2] Fung also observed a similar anisotropy in the T_2 water in frog gastrocnemius muscle (51). We suspect that the observed anisotropy in the relaxation time results from the interaction between water molecules and biomacromolecules (especially the contractile proteins). It would seem that the anisotropic effect should be enhanced in a system in which the water-macromolecular interactions are greatest. It is known that cellular water enters a supercooled phase when the tissue is cooled below 0°C.[3] At lower temperatures, when most of the tissue water is frozen, there is still a fraction of water that does not freeze. This nonfrozen water is generally thought to be hydration water, and the changes in freezing properties are attributed to the water-macromolecular interaction. The nonfrozen water thus offers a convenient system for studying the detailed mechanisms of the interaction between the water molecules and the protein surface. In hopes of gaining further insight into the nature of the anisotropy in the relaxation times and its implications for the ordering of water molecules in muscle, we have carried out an investigation of this anisotropy at three different temperatures (28, −5, and −10°C).

METHODS

NMR Measurements

The spin-lattice relaxation time (T_1) and spin-spin relaxation time (T_2) of the intracellular water protons were measured using a Bruker SXP spectrometer (Bruker Instruments, Inc., Billerica, Mass.) equipped with a frequency synthesizer and a 12-in. electromagnet which has a 1.75-in. air gap. Measurements of T_1 and T_2, were made at room temperature, at −5°C, and at −10°C using a homemade temperature controller. The spectrometer was operated at a frequency of 30.3 MHz in this study. The T_1 was measured using the 180°-τ-90° pulse sequence (54) and by monitoring the full recovery of the magnetization to equilibrium value. The T_2 was measured using the Carr-Purcell-Meiboom-Gill (CPMG) method (55). The 2τ spacing was chosen between 6 ms (at room temperature) and 0.8 ms (at $T = -10$°C). All data of relaxation measurement, both T_1 and T_2, were analyzed using a program based on an iterative curve fitting technique (15). The results reported in this paper are the initial relaxation times which are determined from the initial decay of the magnetization signal. ("Initial decay" means the tangential line of the spin-echo decay curve at $t = 0$. Usually the first 20% of the echo decay curve is linear and can be used for the initial decay fit). The linearity of the detection system was carefully compensated, and all data were normalized by computer against a standard calibration curve. The temperature of the sample could be measured and maintained to an accuracy of 0.5°C.

All T_2 measurements were signal-averaged for 32 repetitive scans. At room temperature, the signal-to-noise ratio was better than 2,000 to 1. At subzero temperature, the signal-to-noise ratio was reduced to ~500 to 1 (due to freezing). The number of repetitive scans in T_1 measurements was eight. The signal-to-noise ratio was similar to that of the T_2 measurements, within a factor of 2. Although our magnetic field was not locked, it drifted less than 100 Hz in 30 min. Since we used diode-detection in all measurements and our radio frequency pulses had an equivalent bandwidth of 100 kHz, this small field drift did not result in any observable instability in the measured signal.

Sample Preparation

The skeletal muscles used in this study were obtained from white rats (Texas Inbred Mouse Company, Houston, Tex.) weighing between 260 and 530 g. The rats were killed by cervical fracture, the tibialis

[2]Chang, D. C., C. F. Hazlewood, and D. E. Woessner. Unpublished data.

[3]Fogal, J. M., D. C. Chang, C. F. Hazlewood, and H. E. Rorschach. Manuscript to be submitted for publication.

anterior muscle was quickly excised, and the gross connective tissue was removed from the tissue. The muscle orientation (θ), which is the angle of the fibers, with reference to the direction of the static magnetic field, was managed as follows (24): a piece of 6-mm (i.d.) glass tubing was attached perpendicular to a glass rod, and this system was placed inside a 10-mm o.d. NMR glass tube so that the muscle sample, held in the 6-mm glass tubing, fitted flush inside the 10-mm o.d. glass tube. The excised muscle was drawn through the 6-mm tube until a linear portion of the muscle was held in place, and the excess portion of the muscle was then trimmed off at both ends. This sample was then placed in the 10-mm o.d. NMR glass tube mentioned above. The orientation of the muscle fiber could be noted at the top of the 10-mm glass tube and was varied by manually rotating the 10-mm glass tube. The measurements were started within 5 to 15 min after the sample was excised. Some samples were stored overnight in the refrigerator; no effect of this procedure on anisotropy was observed.

Sample Cooling

Each muscle preparation was cooled by blowing cold nitrogen gas onto the sample. Most of the samples were cooled by cooling the sample from 0 to −20°C within 30 to 45 min. Some samples (as marked in the results) were cooled at slower rates. For these samples, a minimum of 2 h was required to reach the desired temperature between 0 and −20°C. To insure that most of the tissue was frozen, the sample was first cooled to −20°C (nucleation occurs at $T \simeq -14$°C) and then brought back to the desired

TABLE I

ANGULAR DEPENDENCE OF THE T_2 OF CELLULAR WATER PROTONS AT 28°C IN SKELETAL MUSCLE. ALSO SHOWN IS THE ANGULAR DEPENDENCE OF T_2 IN AGAR GEL SAMPLE UNDER IDENTICAL CONDITIONS

Sample No.	T_2			A_2‡
	$\theta = 0°$	$\theta = 55°$	$\theta = 90°$	
	(*ms*)	(*ms*)	(*ms*)	
1*	39.3 ± 0.3	43.4 ± 0.5	40.1 ± 0.3	1.104 ± 0.015
2	44.6 ± 0.5	45.1 ± 0.6	44.9 ± 0.6	1.011 ± 0.018
3	51.8 ± 0.8	54.4 ± 0.9	53.4 ± 0.7	1.050 ± 0.024
4	47.0 ± 0.4	48.6 ± 0.6	46.9 ± 0.5	1.034 ± 0.015
5	49.3 ± 0.8	50.2 ± 0.9	50.4 ± 0.8	1.018 ± 0.025
6	46.4 ± 0.6	47.7 ± 0.7	47.8 ± 0.7	1.028 ± 0.020
7	45.5 ± 0.6	47.0 ± 0.6	46.3 ± 0.5	1.033 ± 0.019
8	41.3 ± 0.3	42.8 ± 0.3	42.7 ± 0.3	1.036 ± 0.010
9	45.9 ± 0.7	46.7 ± 0.6	45.7 ± 0.5	1.017 ± 0.020
10	42.1 ± 0.4	43.7 ± 0.4	42.9 ± 0.4	1.038 ± 0.013
11	40.8 ± 0.4	44.4 ± 0.5	44.4 ± 0.4	1.088 ± 0.015
12	39.6 ± 0.3	42.7 ± 0.3	43.3 ± 0.4	1.078 ± 0.010
13	42.0 ± 0.6	43.2 ± 0.5	42.5 ± 0.5	1.029 ± 0.019
14	41.9 ± 0.4	44.0 ± 0.5	44.0 ± 0.4	1.050 ± 0.016
15*	48.9 ± 0.6	50.6 ± 0.7	51.1 ± 0.7	1.035 ± 0.019
16	40.2 ± 0.4	44.3 ± 0.5	44.3 ± 0.4	1.102 ± 0.017
17	43.3 ± 0.5	45.6 ± 0.5	44.9 ± 0.4	1.053 ± 0.017
18	41.0 ± 0.4	44.4 ± 0.4	43.0 ± 0.3	1.083 ± 0.014
19	42.0 ± 0.4	45.3 ± 0.4	45.1 ± 0.4	1.079 ± 0.014
20	42.6 ± 0.4	44.0 ± 0.3	44.0 ± 0.3	1.033 ± 0.012
21	41.6 ± 0.5	43.4 ± 0.5	42.8 ± 0.3	1.043 ± 0.017
	$P \ll 0.001$	Mean ± SE		1.050 ± 0.006
22 Agar gel	30.1 ± 0.1	30.0 ± 0.08	29.9 ± 0.1	0.997 ± 0.004

*Sample 1 is EDL muscle and sample no. 15 is soleus muscle.
‡A_2 is defined as $A_2 = T_2(\theta = 55°)/T_2(\theta = 0°)$ (See Text).

temperature. No significant difference was observed between the results obtained using the rapid and the slow cooling methods.

RESULTS

The results of the measurement at room temperature (28°C) of T_2 of tissue water protons in tibialis anterior muscle tissue for $\theta = 0$, 55, and 90° are shown in Table I. Table I also contains one study on the extensor digitorum longus (EDL) muscle and one on the soleus muscle. The results for a model system (3% agar gel sample) are also given in Table I. The measurements for the agar gel sample were conducted under identical conditions using the same sample holder and same size sample, etc. In the last column of this table are the values for the anisotropy parameter, A_2, which we define as follows:

$$A_2 = \frac{T_2(55°)}{T_2(0°)}, \tag{1}$$

where T_2 (55°) and T_2 (0°) are in the spin-spin relaxation times for the muscle orientations θ = 55° and θ = 0°, respectively. An anisotropy parameter, A_1, for the spin-lattice relaxation time, T_1, is similarly defined. The anisotropy parameter is defined as above since one would expect T_2 to be longest for θ = 55° and shortest for θ = 0°, as will be discussed later. The last column of Table I shows a definite anisotropy in every muscle sample investigated. The average value of A_2 is 1.050 ± 0.006, which represents an anisotropy of 5%. These data should be compared

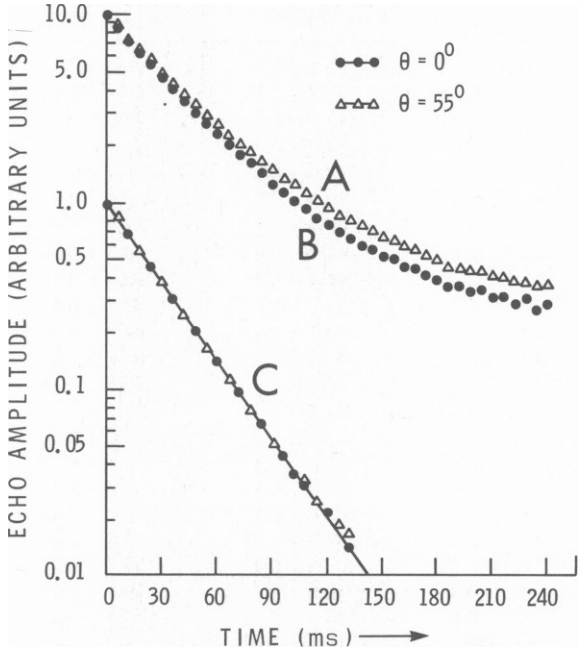

FIGURE 1 Echo decay of water protons using the Carr-Purcell-Meiboom-Gill (GPMG) pulse sequence. θ is the angle between the sample axis and the external magnetic field. (*A*) Muscle sample at θ = 55°. (*B*) Muscle sample at θ = 0°. (*C*) Agar gel sample under identical conditions at θ = 0° and θ = 55°. All the data shown were measured at T = 28°C.

with the datum on the 3% agar gel system in which no anisotropy was observed. A typical proton spin-echo decay at 28°C is shown in Fig. 1. This muscle tissue was measured using a CPMG sequence for the two orientations of 55 and 0°. Also shown in the same figure is the spin-echo decay curve for the water protons in the 3% agar gel sample for the same two orientations. This figure clearly shows that there is no anisotropy in the agar-water model system, while there is an anisotropy in the muscle tissue. The data in Table I were subjected to a statistical test to determine the probability, P, that the mean difference between the T_2 (55°) and T_2 (0°) in the paired observations occurs by chance. For the data in Table I, the probability, P, is much less than 0.001.

Table II shows the T_2 values for muscle protons for different orientations of the muscle fiber at a temperature (T) of −5°C; Table III gives similar results at $T = -10$°C. At $T = -5$°C there is an average anisotropy of ~2%, while at $T = -10$°C, the average anisotropy is 1.3%. A statistical analysis of the data shows that the mean difference between the values of T_2 (55°) and T_2 (0°) in paired observations at $T = -5$°C is statistically significant ($P \ll 0.001$), while at $T = -10$°C, the statistical significance is $0.05 > P > 0.025$. The larger P value observed in the latter case is probably due to the poor signal-to-noise ratio at −10°C. The typical spin-echo decay curves for water protons in the muscle tissue for orientations $\theta = 55°$ and $\theta = 0°$ at −5 and −10°C are presented in Figs. 2 and 3. A small but definite anisotropy in the T_2 is apparent at both temperatures. In the control agar gel sample, no echo signal was observed at these temperatures because the amount of the nonfrozen water in the control sample is extremely small.

TABLE II
ANGULAR DEPENDENCE OF T_2 OF CELLULAR WATER PROTONS IN SKELETAL MUSCLE AT $T = -5$°C

Sample No.	T_2			A_2
	$\theta = 0°$	$\theta = 55°$	$\theta = 90°$	
	(*ms*)	(*ms*)	(*ms*)	
1	5.85 ± 0.04	5.92 ± 0.05	5.94 ± 0.04	1.012 ± 0.011
2	5.40 ± 0.05	5.37 ± 0.03	5.16 ± 0.05	0.994 ± 0.011
3	5.76 ± 0.06	5.83 ± 0.06	5.65 ± 0.05	1.012 ± 0.015
4	6.02 ± 0.10	5.98 ± 0.07	5.84 ± 0.07	0.993 ± 0.020
5	5.15 ± 0.02	5.24 ± 0.03	5.17 ± 0.04	1.017 ± 0.007
6	5.53 ± 0.04	5.82 ± 0.05	5.66 ± 0.04	1.052 ± 0.012
7	5.36 ± 0.07	5.50 ± 0.08	5.50 ± 0.09	1.026 ± 0.020
8	5.67 ± 0.05	5.83 ± 0.05	5.71 ± 0.05	1.028 ± 0.013
9	5.82 ± 0.05	5.92 ± 0.04	5.78 ± 0.05	1.017 ± 0.011
10	5.35 ± 0.08	5.27 ± 0.04	5.17 ± 0.06	0.985 ± 0.017
11	5.77 ± 0.03	5.81 ± 0.03	5.59 ± 0.04	1.007 ± 0.007
12	5.16 ± 0.05	5.33 ± 0.05	5.24 ± 0.05	1.033 ± 0.014
13	6.18 ± 0.04	6.30 ± 0.03	6.19 ± 0.04	1.019 ± 0.008
14*	5.81 ± 0.04	6.08 ± 0.03	6.01 ± 0.04	1.047 ± 0.009
15	6.03 ± 0.04	6.22 ± 0.04	5.98 ± 0.04	1.032 ± 0.010
16*	5.29 ± 0.04	5.57 ± 0.04	5.51 ± 0.04	1.053 ± 0.011
17*	5.50 ± 0.04	5.71 ± 0.05	5.80 ± 0.05	1.038 ± 0.012
		$P \ll 0.01$	Mean ± SE	1.021 ± 0.005

*Samples 14, 16, and 17 have been cooled slowly at the rate mentioned in the text.

TABLE III
ANGULAR DEPENDENCE OF T_2 OF CELLULAR WATER PROTONS IN SKELETAL MUSCLE AT $T = -10°C$

Sample No.	T_2			A_2
	$\theta = 0°$	$\theta = 55°$	$\theta = 90°$	
	(*ms*)	(*ms*)	(*ms*)	
1	3.06 ± 0.03	3.27 ± 0.04	3.05 ± 0.04	1.069 ± 0.17
2	3.42 ± 0.08	3.58 ± 0.09	3.36 ± 0.06	1.047 ± 0.076
3	3.21 ± 0.07	3.16 ± 0.04	3.13 ± 0.05	0.984 ± 0.025
4	3.27 ± 0.07	3.34 ± 0.04	3.25 ± 0.05	1.021 ± 0.029
5	3.02 ± 0.04	3.03 ± 0.04	2.99 ± 0.04	1.003 ± 0.019
6	3.49 ± 0.10	3.56 ± 0.09	3.28 ± 0.07	1.020 ± 0.039
7	2.82 ± 0.04	2.87 ± 0.06	2.80 ± 0.04	1.018 ± 0.026
8	3.17 ± 0.05	3.13 ± 0.05	3.24 ± 0.06	0.987 ± 0.022
9	2.85 ± 0.06	2.80 ± 0.04	2.74 ± 0.05	0.982 ± 0.025
10	3.20 ± 0.04	3.22 ± 0.03	3.19 ± 0.04	1.006 ± 0.016
11*	3.26 ± 0.04	3.33 ± 0.03	3.29 ± 0.04	1.022 ± 0.016
12	3.16 ± 0.05	3.22 ± 0.03	3.21 ± 0.04	1.019 ± 0.019
13*	3.04 ± 0.04	3.07 ± 0.02	3.06 ± 0.04	1.010 ± 0.015
14*	3.06 ± 0.04	3.02 ± 0.07	3.00 ± 0.05	0.987 ± 0.026
	$0.05 > P > 0.025$		Mean ± SE	1.013 ± 0.007

*Samples 11, 13, and 14 are cooled slowly at the rate mentioned in the text.

The results of the measurement of the proton T_1 for muscle orientations $\theta = 0$, 55, and 90° at $T = 28°C$ are shown in Table IV. The anisotropy parameter, A_1, is defined in a way similar to A_2. The results in this table show that the anisotropy is very small (0.7%). The mean difference of the T_1 values at $\theta = 0°$ and $\theta = 55°$ is, however, statistically significant. The

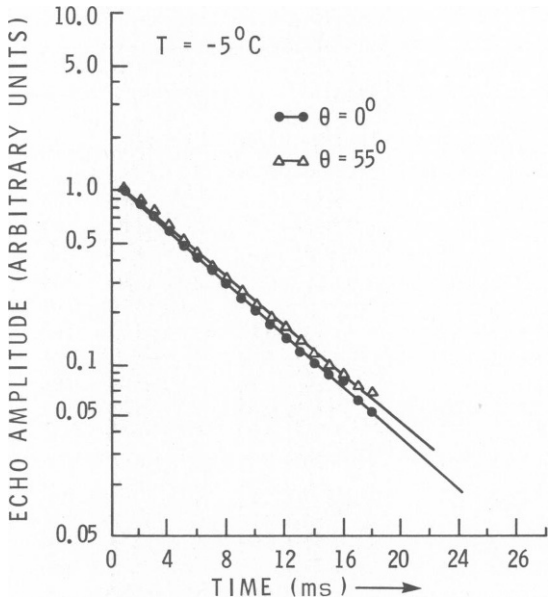

FIGURE 2 Echo decay of cellular water protons in skeletal muscle using the CPMG sequence at $T = -5°C$ for $\theta = 0°$ and $\theta = 55°$.

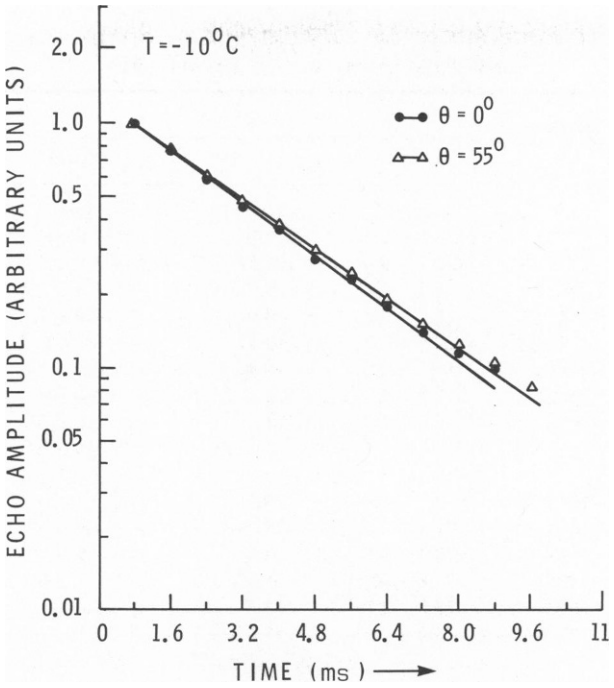

FIGURE 3 Echo decay of cellular water protons in skeletal muscle using the CPMG sequence at $T = -10°C$ for $\theta = 0°$ and $\theta = 55°$.

angular dependence of the T_1 data at -5 and $-10°C$ is summarized in Table V. At $T = -5°C$, the mean difference between T_1 (55°) and T_1 (0°) is not statistically significant ($P > 0.5$); the results are similar for $T = -10°C$ ($0.5 > P > 0.25$). The average anisotropy at these two temperatures is 0.2% and is within experimental error.

TABLE IV
ANGULAR DEPENDENCE OF T_1 OF CELLULAR WATER PROTONS IN SKELETAL MUSCLE AT 28°C

Sample No.	T_1			A_1
	$\theta = 0°$	$\theta = 55°$	$\theta = 90°$	
	(*ms*)	(*ms*)	(*ms*)	
1	668 ± 2	673 ± 5	674 ± 3	1.008 ± 0.008
2	636 ± 1	636 ± 2	636 ± 1	1.000 ± 0.004
3	705 ± 1	713 ± 1	713 ± 1	1.011 ± 0.002
4	704 ± 1	704 ± 1	705 ± 1	1.000 ± 0.002
5	685 ± 1	693 ± 1	691 ± 1	1.012 ± 0.002
6	675 ± 1	677 ± 1	678 ± 1	1.003 ± 0.002
7	658 ± 1	666 ± 1	665 ± 1	1.012 ± 0.002
8	652 ± 1	652 ± 1	651 ± 1	1.000 ± 0.002
9	662 ± 1	670 ± 1	674 ± 1	1.012 ± 0.002
10	668 ± 1	675 ± 1	674 ± 1	1.010 ± 0.002
	$0.005 > P > 0.001$		Mean ± SE	1.007 ± 0.002

*A_1 is defined as $A_1 = T_1\ (\theta = 55°)/T_1\ (\theta = 0°)$.

TABLE V
ANGULAR DEPENDENCE OF T_1 OF CELLULAR WATER PROTONS IN SKELETAL MUSCLE AT −5 and −10°C

Sample No.	Temperature (T)	T_1			A_1
		$\theta = 0°$	$\theta = 55°$	$\theta = 90°$	
		(*ms*)	(*ms*)	(*ms*)	
1	−5°C	120.0 ± 0.5	122.0 ± 0.6	122.0 ± 0.6	1.017 ± 0.006
2	−5°C	122.2 ± 0.6	122.5 ± 0.7	123.9 ± 0.4	1.002 ± 0.008
3	−5°C	114.2 ± 1.6	113.9 ± 1.3	114.6 ± 1.4	0.997 ± 0.018
4	−5°C	115.5 ± 0.7	114.9 ± 0.9	114.9 ± 1.1	0.995 ± 0.010
5	−5°C	120.9 ± 0.7	119.1 ± 1.1	118.8 ± 1.0	0.985 ± 0.011
6	−5°C	118.5 ± 1.0	119.4 ± 0.6	120.3 ± 1.0	1.008 ± 0.010
7	−5°C	113.4 ± 1.3	113.1 ± 1.7	114.6 ± 1.6	0.997 ± 0.019
8	−5°C	120.9 ± 0.8	121.0 ± 0.7	121.8 ± 0.7	1.001 ± 0.009
			$P > 0.5$	Mean ± SE	1.002 ± 0.003
1	−10°C	78.7 ± 0.6	79.0 ± 0.8	79.3 ± 0.7	1.004 ± 0.013
2	−10°C	74.8 ± 1.2	75.2 ± 0.8	74.9 ± 0.9	1.005 ± 0.019
3	−10°C	78.3 ± 0.8	78.4 ± 0.9	77.9 ± 1.0	1.001 ± 0.015
4	−10°C	75.8 ± 0.8	75.8 ± 1.0	76.9 ± 0.7	1.000 ± 0.017
5	−10°C	72.8 ± 1.9	73.3 ± 1.5	73.8 ± 1.5	1.007 ± 0.033
6	−10°C	76.0 ± 1.3	75.6 ± 1.3	76.1 ± 1.2	0.995 ± 0.024
			$0.5 > P > 0.25$	Mean ± SE	1.002 ± 0.0018

DISCUSSION

Although a very small anisotropy in the T_1 tissue water protons in tibialis anterior muscle tissue was observed at room temperature, none was observed at the subzero temperatures. A definite anisotropy in the T_2 of water protons in the muscle tissue, however, was observed. The origin of the anisotropy has been thought to be due to the partial orientation (ordering) of the water molecules as they interact with the actin-myosin filaments (44, 47–51). In broad line NMR studies of hydrated collagen (44, 46) and oriented DNA (49), the angular dependence of the dipolar splitting was found to obey the angular relationship

$$\Delta B = K(3\cos^2\theta - 1), \tag{2}$$

where ΔB is the separation of the resonance peaks, K is a constant which depends on sample hydration, and θ is the angle between the sample fiber axis and the direction of the static field. Since the effective T_2 is in general inversely proportional to the dipolar splitting, one may expect that the average T_2 will be longest for $\theta = 55°$ and shortest for $\theta = 0°$, with the T_2 value for $\theta = 90°$ lying in between. In tibialis anterior muscle fiber, this is precisely what was observed for T_2 at 28°C. At −5 and −10°C, a similar angular dependence was found, although it was not as well defined as that at 28°C. These observations suggest the possible existence of water chains along the direction of the muscle fiber (44) or, alternatively, the existence of an anisotropic reorientation of H_2O mulecules (46, 50).

The preferred orientation of water molecules may or may not be localized. The bulk of the myoplasmic water may be "ordered" to some extent, or, only a small part of the water bound to the surface of the biomacromolecules may be preferentially oriented, but due to an exchange between the "bound" water and the bulk water in the tissue, a new anisotropy of the

TABLE VI

TEMPERATURE DEPENDENCE OF ISOTROPIC AND ANISOTROPIC CONTRIBUTIONS TO TRANSVERSE RELAXATION RATE

Temperature	$\left\langle \frac{1}{T_2(55°)} \right\rangle_{av}$	$A_2 - 1$	$\frac{1}{T_{2,aniso}}$	$*P/T_{2,aniso}$
(°C)	(s^{-1})		(s^{-1})	
28	21.9	0.050 ± 0.006	1.1 ± 0.1	1.1
−5	174.5	0.021 ± 0.005	3.7 ± 0.9	0.93
−10	315.4	0.013 ± 0.007	4.1 ± 2.2	0.82

*P is normalized to 1.0 at 28°C.

average T_2 is observed. If the latter possibility were true for muscle water, then one would expect that a larger anisotropy of T_2 at temperatures below 0°C would have been observed, and the anisotropy would have increased as the temperature was lowered, because the isotropically rotating bulk water molecules have the least interaction with muscle proteins and would have frozen at subzero temperatures. We found, however, that the anisotropy decreased as the temperature was lowered. This observation may be explained in two ways; (*a*) the preferred orientation is not localized in the nonfrozen region, or (*b*) the isotropic relaxation rate increases faster than the anisotropic relaxation rate as the temperature is decreased. These two interpretations are not mutually exclusive, and we found evidence that both of them are operative to a certain extent. The T_2 anisotropy, for example, can be analyzed by noting that the T_2 depends both on isotropic and anisotropic terms:

$$\frac{1}{T_2} = \frac{1}{T_{2,iso}} + \frac{1}{T_{2,aniso}}. \tag{3}$$

Furthermore, Eq. 2 suggests that $1/T_{2,iso} = 1/T_2(55°)$. Hence,

$$\frac{1}{T_{2,aniso}} = \frac{1}{T_2(0°)} - \frac{1}{T_2(55°)}$$

$$= (A_2 - 1)\frac{1}{T_2(55°)},$$

and the results in Table VI show that while $1/T_{2,iso}$ increases rapidly as temperature decreases, $1/T_{2,aniso}$ increases also, but by a much smaller percentage.

If the anisotropic relaxation rate is attributed to the exchange of the bulk water with a very small amount of bound water, we may write[4]

$$\frac{1}{T_{2,aniso}} = \frac{b}{P}\frac{1}{T_{2b}}, \tag{4}$$

where b is the amount of "bound" water which has preferred orientation. P is the amount of exchangeable water and T_{2b} is the relaxation time of the bound water. At room temperature, P

[4]A general expression of Eq. 4 may include τ (the exchange time), i.e., $1/T_{2,aniso} = (b/P)/(1/T_{2b} + \tau)$. However, since the T_2 decay at subzero temperatures approaches a simple exponential function, it mat be concluded that τ is much shorter than T_{2b} and thus can be omitted.

equals the amount of total water. At lower temperatures, P equals the amount of nonfrozen water which is 25 and 20% of total water at -5 and -10°C, respectively.

The value of $P/T_{2,\text{aniso}}$ is given in the last column of Table VI. According to Eq. 4

$$\frac{P}{T_{2,\text{aniso}}} = \frac{b}{T_{2b}},$$

If b is less than the amount of nonfrozen water and, therefore, is not affected by freezing, then $P/T_{2,\text{aniso}}$ should be constant or increase with decreasing temperatures since (T_{2b} should be shortened as temperature decreases). However, we find experimentally that $P/T_{2,\text{aniso}}$ decreases when the muscle freezes. This implies that b must decrease with decreasing temperature. That is, part of the preferentially oriented water freezes when the muscle sample is frozen. This result suggests that the preferred orientation is a rather long-range effect which extends outside of the hydration (nonfrozen) water layer.

Relatively long-range preferred ordering in water molecules has been proposed for some NMR studies of biological systems (46, 56). Woessner and Snowden (56), for example, have observed domains of long-range ordering of polymer molecules of size as large as 15 μm in which water molecules are preferentially oriented.

The data in Table VI show that the isotropic relaxation rate increases by a factor of 15 when the temperature is decreased from 28 to -10°C. This increase of relaxation rate may result from the following causes: (*a*) The nonfrozen water is the hydration water of proteins. Therefore, both the inter- and intramolecular correlation times of these water protons are much longer than that of the total cytoplasmic water (57, 58). (*b*) The T_2 of the cytoplasmic water could be shortened by proton exchange between water and macromolecules (59). Because of an increase in the surface-to-volume ratio at freezing, this proton exchange mechanism would be more effective at subzero temperature. (*c*) The bulk water freezes into ice at subzero temperatures and its relaxation rate is greatly increased. The proton exchange between the nonfrozen water and ice could also increase the relaxation rate of the nonfrozen water. It seems likely that all of these causes could be operative to some extent and it may well be a combination of these effects which brings about the reduction of the isotropic T_2. A detailed investigation of the contribution of these effects may help to gain some insight into the structure and properties of hydration water.

Another possible cause of the shortening of T_2 at subzero temperatures is that mobile organic molecules may contribute significantly to the proton NMR signal (25, 26). Fung (27) estimated that protons from mobile organic molecules amount to 6–8% of the total nonrigid protons in muscle, and that their T_2 at 27°C could be of the order of 0.4–2 ms. At temperatures below the freezing point, their contribution to the total NMR signal would be quite significant, and their T_2 would exhibit no angular dependence. Consequently, the resultant anisotropy in the T_2 data would decrease. On the basis of this argument, the anisotropy should decrease with a decrease in temperature. Berendsen (44) observed a narrow absorption peak in the H_2O spectra of collagen hydration, which Dehl and Hoeve (46) showed was not exchangeable with water. This signal could be due to the mobile organic molecules. However, the amount of mobile protons involved is relatively small (roughly 3% of the total macromolecular protons). We believe that Fung's estimate of the concentration of protons from mobile organic molecules is too high. Since the amount of dry solids in a cell is only

roughly 20%, Fung's estimate would imply that almost 30% of the dry solid in the muscle would give a mobile proton signal. The available data, in our opinion, do not support Fung's interpretation.

We observed very little anisotropy (<1%) in the proton spin-lattice relaxation data in muscle tissue either at 28°C or at temperatures below 0°C. Nor did Fung (51) observe any angular dependence of T_1 for ^1H and ^2H in frog gastrocnemius muscle at room temperature. The absence of T_1 anisotropy may indicate that the preferred orientation is associated with the low frequency reorienting motion of the water molecules. It is well known that T_1 is affected mainly by the motion having a frequency comparable to the Larmor frequency, while T_2 is most sensitive to fluctuations in the local fields at low frequency. Our findings would suggest that the anisotropic motion of water molecules must be concentrated in the low frequency range [$\tau_c \gg (1/\omega_0) \sim 10^{-8}$ s].

In conclusion, we have made a quantitative determination of the degree of anisotropy for the bulk of the myoplasmic water as well as for the water that remains unfrozen at temperatures below 0°C. These results demonstrate that a definite but small anisotropy ($\simeq$5%) exists in the spin-spin relaxation times of water protons in tibialis anterior muscle at room temperature. We find that the anisotropy decreases to 2.1% at −5°C and 1.3% at −10°C. These findings suggest that the bulk of the myoplasmic water has a small degree of ordering due to the anisotropic interaction between the water molecules and cellular macromolecules (presumably the actin-myosin filaments).

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