A STUDY OF MOLECULAR DYNAMICS AND FREEZING PHASE TRANSITION IN TISSUES BY PROTON SPIN RELAXATION

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ABSTRACT Muscle, spleen, and kidney tissues from 4-wk-old C57 black mice were studied by proton magnetic resonance. Spin-lattice relaxation times at high fields and in the rotating frame, as well as the spin-spin relaxation times, are reported as a function of temperature in the liquid and frozen phase. Motions of large molecules and of water molecules and their changes at the freezing phase transition are studied. The shortcomings of the two-state fast-exchange relaxation model are discussed.

INTRODUCTION

Most tissues of mice freeze at around $-8°C$. In this process up to 90% of water molecules instantaneously crystallize (1,2). The size and shape of ice crystallites depend on the cooling rate at which the freezing temperature has been reached. Since this rate is difficult to monitor with precision, information on the establishment of the frozen phase is mainly qualitative. For example, it is known that during slow cooling large, round ice crystallites will form, while a fast cooling rate gives rise to numerous small, pointed ice crystals. Considerable indirect information is available from studies of cell survival at various programmed cooling rates (3,4).

A study of the hydration of macromolecules in solution has shown (5,6) that even at $-40°C$ a fraction of water proportional to the concentration of macromolecules does not freeze. It has been observed (1,2) that at such a temperature a small fraction ($\sim 10\%$) of water in tissues also remains in a liquid-like state: the so-called "nonfreezable" water. The changes that occur in the mobility of large molecules (we use the word large molecule for all molecules but water) at the freezing phase transition, and the transformation of the waxy phase of large molecules coated by a monolayer of the nonfreezable water into a glassy phase, are by and large not known. In this study the freezing phase transition is investigated by examining its effect on molecular dynamics. The experimental method used is nuclear magnetic resonance. The molecular motions contributing to spin relaxation (1,2,7–33) are analyzed, and the freezing transition, as

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well as the waxy (34) to glassy transition, for the bound water-biopolymers mixture are discussed. Some observations on frozen tissues deuterated under isotonic (phosphate-buffered saline) conditions are also reported.

METHODS

A CP-2 Spin-Lock spectrometer (Spin-Lock Ltd., Port Credit, Ontario, Canada) operating at 33.8 MHz was used for measurements of spin-lattice relaxation time T_1, spin-lattice relaxation time in the rotating frame $T_{1\rho}$, and spin-spin relaxation time T_2. The relaxation times T_1 and $T_{1\rho}$ were determined by the 90° – 90° and 90°—spin-locking field pulse sequences, respectively. T_2 was determined by the modified Carr-Purcell sequence above −20°C and was estimated from the free induction decay (FID) shape below this temperature. The dispersion of T_1 was measured with a Bruker pulse spectrometer (Bruker Instruments, Inc., Billerica, Mass.). If the magnetization recovery was not exponential, the relaxation time is given as the time in which magnetization recovers to $1/e$ of its original value. In the spin-locking experiment, in which the magnetization decay was always nonexponential, $T_{1\rho}$ was set to be the time in which the magnetization decays to $1/e$ of its initial (nonequilibrium) value.

4-wk-old C57 black mice were used throughout this study. The tissue samples were blotted free of blood, cut into ~0.1 cm^3 pieces, and placed in a 20-mm-long glass tube of 6 mm diameter, which was sealed with epoxy resin. The samples were obtained from Dr. W. R. Inch, Ontario Cancer Foundation, Victoria Hospital, London, Ontario.

The osmotically balanced (isotonic) deuteration was achieved by immersion in a phosphate-buffered saline (PBS) 99% D_2O solution. The samples were immersed twice in this solution, allowing 4 h for each exchange.

RESULTS

Nuclear magnetic resonance experiments on tissues were performed in the liquid-like phase I, across the freezing transition (T_c) and in the frozen "waxy" (34) phase II from T_c to ~ −90°C, as well as below this temperature (T_g), when tissues become "glassy," phase III.

Phase I

It was proposed (20) that the dispersion in tissues observed in rotating frame experiments was primarily due to the exchange diffusion of water molecules between the "bound" and the "free" state. To support this interpretation of the low field relaxation, dispersion measurements of $T_{1\rho}$ between 10 and 1 G at 297°K for protonated, partially deuterated (60% D_2O – 40% H_2O), and totally deuterated muscle were undertaken, (Fig. 1). The strong dispersion of protonated muscle (Fig. 1, lower part) is retained in the partially deuterated sample. In this sample the intra H_2O proton interactions are considerably reduced (16% H_2O, 36% D_2O, 48% HDO) and yet the dispersion of $T_{1\rho}$ is almost as strong as in the fully protonated sample. In the totally deuterated sample almost no dispersion is seen.

Fig. 2 shows the high-field dispersion (20–60 MHz) for protons in fully deuterated tissue. It can be seen that relaxation processes of protons on large molecules include a slow mechanism for which $\omega_L \tau > 1$.

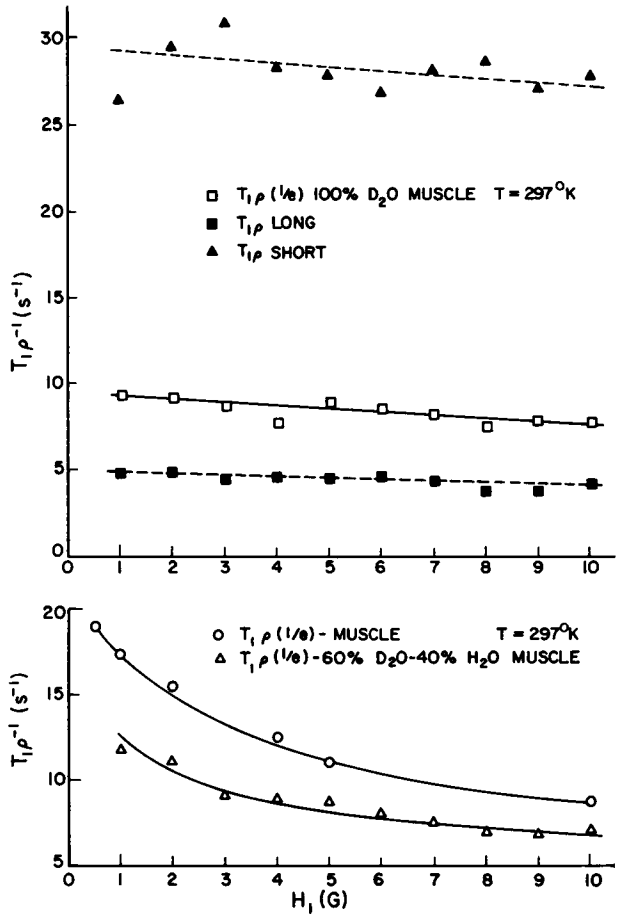

FIGURE 1 Dispersion of $T_{1\rho}$ in 100% D_2O muscle, 60% – 40% H_2O muscle, and natural muscle at ν_0 = 33.8 MHz. The dashed lines in the upper figure represent the components obtained from a graphical decomposition of the nonexponential decay curves.

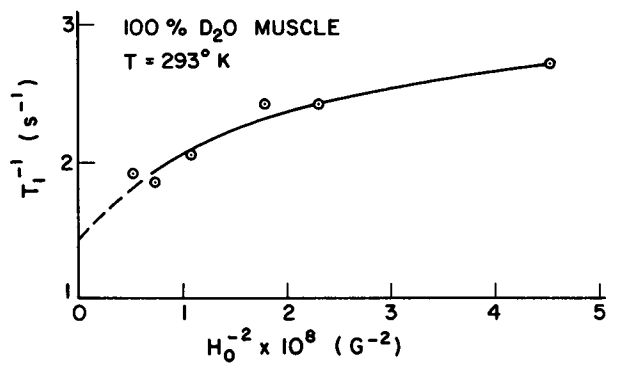

FIGURE 2 T_1^{-1} versus H_0^{-2} for 100 D_2O muscle.

Proton T_1^{-1} in the natural muscle tissue has been measured (20) as a function of frequency over the range 17–45 MHz and also recently (33) over the frequency range 10^4–10^8 Hz. In this study the T_1^{-1} dispersion of protons over the frequency range 15–50 MHz at 293 and 273°K has been measured in pure and 60% D_2O muscle tissue. It is most significant that the dispersion of partially deuterated samples is not substantially different from dispersion of natural muscle tissue. It is not possible to differentiate, within the accuracy of a computer-simulated extrapolation, between dispersions in the natural and partially deuterated muscle tissue. The totally deuterated muscle T_1^{-1} is also dispersive, but to a lesser extent. The 60% D_2O and the natural tissues have correlation times at 293°K equal to 4 ± 7 and $6 \pm 7 \times 10^{-9}$ s, respectively.

Phases II and III

In Figs. 3 and 4, the temperature dependence of ice proton T_1 at 33.8 MHz in muscle is shown. For comparison, results at 30 MHz from pure ice crystal are also given (35). Pure ice T_1 at 33.8 MHz would be larger than T_1 of ice in a tissue at 30 MHz (dotted line in Fig. 3) by a factor of 1.27. The experimental data, however, show that proton T_1 of ice crystals in muscle is shorter by a factor of two to four in the range -5 to -35°C, with a slope of only 9.0 ± 0.7 as opposed to 14.3 kcal/mol in pure ice.

In phase II and III of the muscle tissue T_1 shows two, and $T_{1\rho}$ three, minima (Fig. 5).

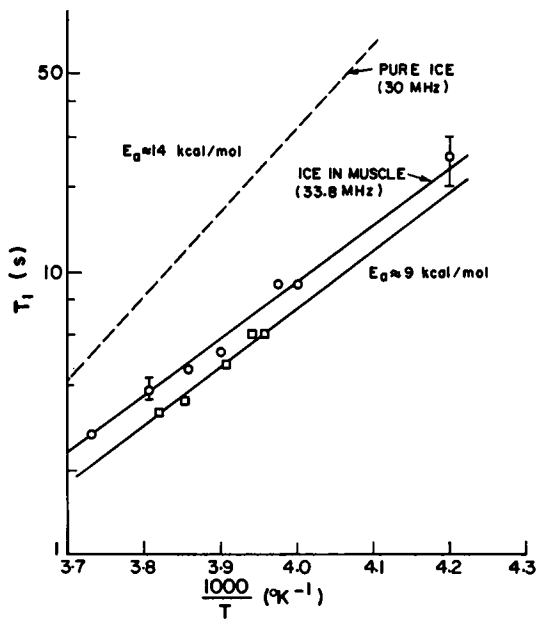

FIGURE 3 T_1 comparison of pure ice and ice in muscle tissue. T_1 in pure ice is that given in ref. 35.

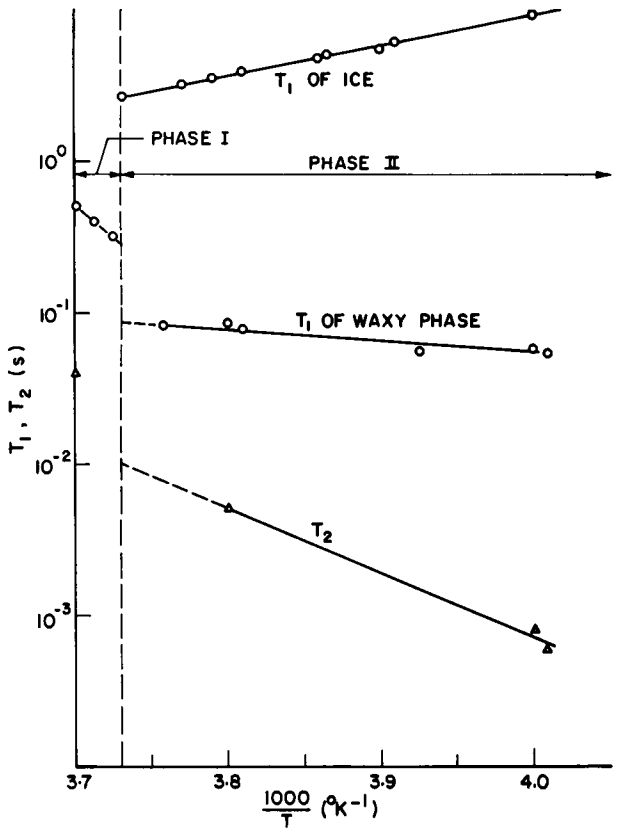

FIGURE 4 T_1 and T_2 across the phase I-phase II transition.

All other tissues, as well as muscle tissue in a deionized state, show fairly similar behavior (see Figs. 6, 7, and 8, also Table I). The deuterated muscle tissue, however, is distinctly different (Fig. 9 and Table I).

The FID in fully deuterated muscle consists of two distinct components in the temperature range from room temperature to approximately 210°K. The slow-decaying part of the FID has a discontinuous T_1 ($1/e$ value) at T_c, whereas the fast-decaying part has T_1 ($1/e$ value) that is essentially continuous.

In the temperature range from room temperature to T_c, measurements were performed at a 200-μs window following the 90° pulse or the field pulse trailing edge, for T_1 and $T_{1\rho}$ measurements, respectively. Below T_c a 15-μs window was used for these measurements, which corresponds to readings taken on the fast-decaying part of the FID.

The dashed lines in Figs. 5–9 represent T_2 below about 30 μs estimated from the fraction of the FID not obscured by the dead time of the spectrometer ($\sim 8\ \mu$s).

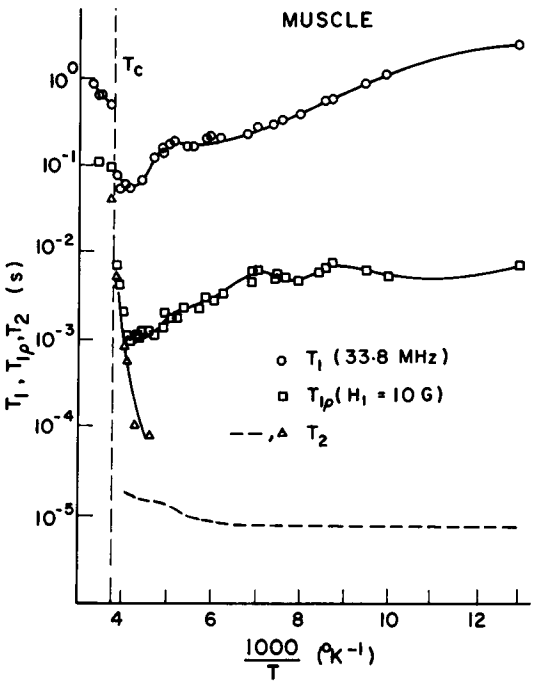

FIGURE 5

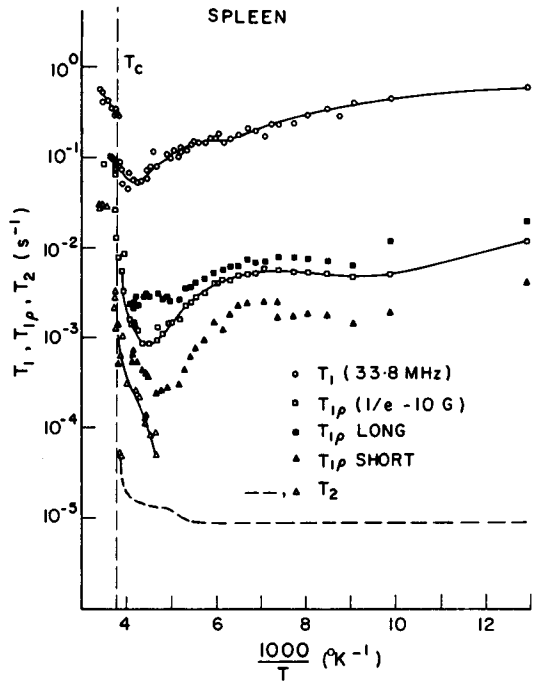

FIGURE 6

FIGURE 5 Relaxation times as a function of inverse temperature in natural muscle.
FIGURE 6 Relaxation times versus inverse temperature in natural spleen.

DISCUSSION

Considerable experimental nuclear magnetic resonance information is available for tissues (1,2,7–33) and protein systems (36–42) in phase I. While the origin of spin-spin relaxation is not yet completely understood, the processes responsible for the spin-lattice relaxation are understood better. It was proposed (29) that the observed high-field water proton Zeeman relaxation rate of a compartment "i" is a weighted average of the relaxation rate of water in the "bound" and "free" states:

$$[1/T_1]_i = b_i[1/T_{1b}]_i + (1 - b_i)1/T_{1f}. \quad (1)$$

The condition of fast exchange is implied in this equation. The translational (exchange) diffusion, which averages the proton spins over sites b and f, is distributed over a large frequency range and has a considerable part at very low frequencies. Even its low frequency component is fast enough to satisfy the fast exchange condition because of long T_1. This low-frequency part of the exchange frequency distribution has the correlation time τ_d very long: $\tau_d\omega_L \lesssim 10^4$. While $1/T_{1f}$, which is less than 1 s^{-1}, is assumed to be the same in all intra- and extracellular compartments, the rate $[1/T_{1b}]_i$ is different in different compartments (29). The compartment "i" is a small volume of water and large molecules within which boundaries water (exchange) diffu-

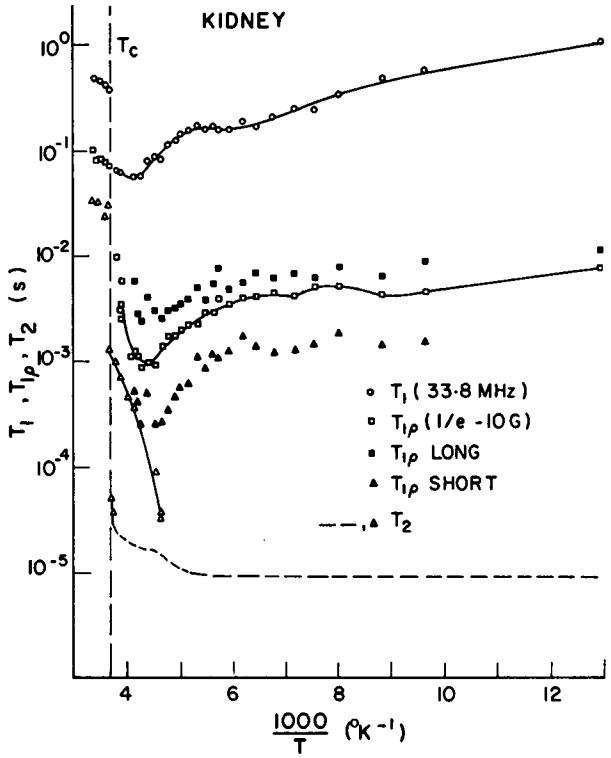

FIGURE 7 Relaxation times versus inverse temperature in natural kidney.

sion mixes b and f states strongly. The bound water abundance, b_i, varies among compartments. However, since it is an experimental fact (18,20,25) that the water proton relaxation is accidentally exponential, the difference in $[1/T_1]_i$ among various compartments could not be very large. For this reason the index "i" in Eq. 1 is dropped. $1/T_{1b}$ and b then represent averages over all compartments. A similar discussion, with two distinctions, applies to the Zeeman relaxation in the rotating frame. First, the slow exchange diffusion contributes to the relaxation directly. Second, the condition of "fast exchange" does not hold as strongly as at high fields because $T_{1\rho}$ is much shorter than T_1. As a consequence, the decay of the magnetization in the rotating frame is much less exponential. In the solid phases II and III the decay of the magnetization becomes distinctly nonexponential.

The dispersion results in the rotating frame of natural and partially deuterated tissues clearly demonstrate that most of the dispersion in this region is the result of intermolecular relaxation generated by slow diffusion. Such slow diffusion could only be translational motion between "bonded" and "free" state (exchange diffusion) or translational motion within the bonded layer, or both. The observation that the 100% deuterated sample shows almost no dispersion in the rotating frame could only mean that for motions of large molecules, $\omega_1 \tau < 1$ at 1 G. It is of course possible that there

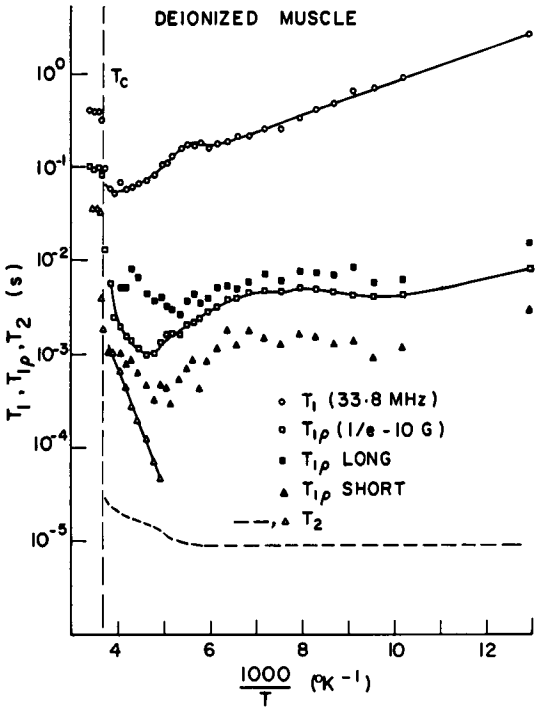

FIGURE 8 Relaxation times versus inverse temperature in deionized muscle. To deionize the tissue it was immersed twice in distilled H_2O, each immersion lasting for 4 h.

TABLE I

TISSUE PROTON T_1 AND $T_{1\rho}$ MINIMA IN PHASES II AND III.

Tissue	$T_{1\delta}$	(Temp.)	$T_{1\rho\alpha}$ (Temp.)						$T_{1\gamma}$	(Temp.)	$T_{1\rho\gamma}$	(Temp.)
			1/e value		Short comp.		Long comp.					
	ms	°K	*ms*	°K	*ms*	°K	*ms*	°K	*ms*	°K	*ms*	°K
Muscle (Fig. 5)	60	(233)	1.0	(227)					200	(167)	5.0	(91)
		4.3		4.4						6.0		11.0
Spleen (Fig. 6)	53	(238)	0.83	(222)	0.26	(208)	2.7	(196)	160	(161)	4.5	(108)
		4.2		4.5		4.8		5.1		6.2		9.3
Kidney (Fig. 7)	60	(238)	0.90	(227)	0.24	(217)	3.0	(208?)	160	(167)	4.2	(111)
		4.2		4.4		4.6		4.8?		6.0		9.0
Deionized (hypotonic) H_2O muscle (Fig. 8)	55	(250)	1.0	(213)	0.40	(200)	2.7	(185)	160	(167)	4.0	(102)
		4.0		4.7		5.0		5.4		6.0		9.8
PBS (isotonic)	Not seen		Not α									
D_2O muscle (Fig. 9)			5.6	(244)					130	(169)	3.9	(102)
				4.1						5.9		9.8

*Numbers in parentheses are temperatures. The Larmor frequency is 33.8 MHz and the radio frequency field H_1 is 10 G. For comparison with figures the inverse temperature (β) values are given below each temperature.

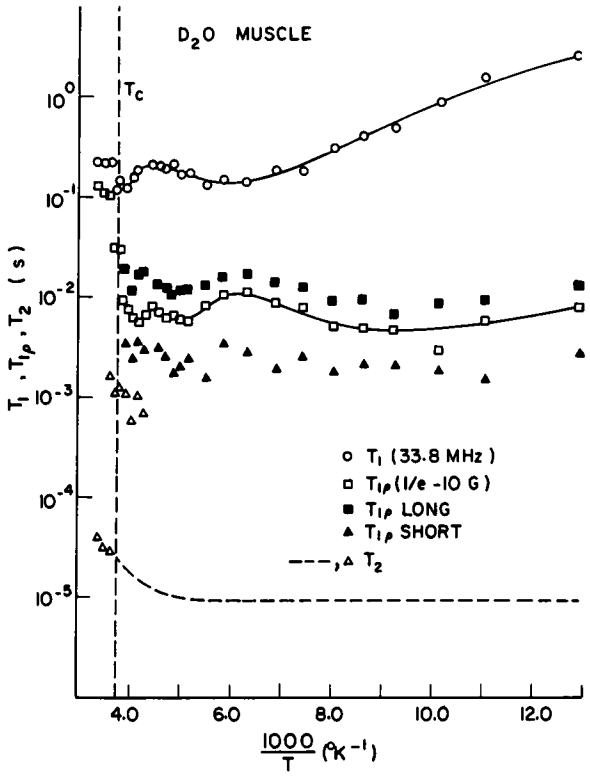

FIGURE 9 Relaxation times versus inverse temperature in 100% D_2O muscle.

is a still slower mode of large molecules, such that $\omega_1 \tau \gg 1$ at less than 1 G. This possibility seems remote, however. That a partially deuterated sample shows no dispersion for deuteron resonance below $\sim 10^5$ Hz (33) is in complete accord with the above observations.

It should be mentioned also that in the muscle tissue the nonexponentiality of the magnetization decay after a spin-locking pulse increases with deuteration. In the protonated sample, where most of the protons are H_2O protons, the nonexponentiality is small. In the partially deuterated sample it is quite pronounced and it is very strong in the totally deuterated sample (Fig. 1, upper graph). To illustrate this point, the nonexponential decay curve was graphically decomposed into fast- and slow-relaxing components. These are approximately 25% and 75% of the total magnetization, respectively. The middle line in Fig. 1, upper part, is the dispersion of the "effective" relaxation time (in which the magnetization decays to $1/e$ of its initial value). In a similar way the magnetization decay was analyzed for all samples below T_c. It must be noted that such a decomposition is not unique and the results should be used with care.

The increase of nonexponentiality with deuteration is expected. The water relaxation in tissues is fairly similar in most compartments. On the other hand, the large

molecules have proton groups which are in substantially different surroundings and are not being averaged by spin diffusion, nor by exchange diffusion. As a result, the relaxation times differ by as much as an order of magnitude, (Fig. 1, upper graph). In a protonated sample the magnetization resulting from large molecules represents only $\sim 15\%$ of the total magnetization; thus its nonexponential behavior is, to a large degree, weighted out.

It is surprising, in view of the recently proposed intra-H_2O origin of the low-field dispersion (24,31), that the relaxation rates of natural muscle tissue and of partially deuterated muscle differ so little. This means that the intra-H_2O reorientation contributes no more than $\approx 3\ s^{-1}$ to the total rate in this frequency range. This observation implies that the relaxation rate of inter-H_2O (exchange) diffusion is very important and that it is spread over a wide range: from 20 kHz, where it shows dispersion in $T_{1\rho}$, to 30 MHz. It has been shown (20) that the proton relaxation rate is dispersive in the frequency range 10–50 MHz. This was attributed to the intramolecular reorientation for which $\omega_L \tau_R \gtrsim 1$. It is proposed that the intermolecular (exchange) diffusion also contributes to this dispersion.

The facts that natural and partially deuterated muscle tissues have the same dispersions within the accuracy of the experiment, and that the 100% deuterated muscle tissue also shows considerable dispersion can be explained as follows. In the totally deuterated muscle the relaxation rate of protons on large molecules only is observed. If the protons in muscle tissue would relax mainly by water reorientation (an intramolecular process), relaxation rates in "60% D_2O" tissue would be smaller by close to a factor of five in comparison with the nondeuterated sample. Since the relaxation rates of partially deuterated tissue are smaller by only a factor of two from natural tissue, it must be that an intermolecular relaxation process, such as translational (exchange) diffusion, is also contributing in this range. Thus, the (exchange) diffusion extends over four orders of magnitude in correlation time and is a contributing relaxation mechanism also at high fields.

In Fig. 2 it is shown that the relaxation processes of protons on large molecules include a slow mechanism such that $\omega_L \tau > 1$. Fast relaxation is experienced by protons of end groups (like CH_3). Possibly some smaller side-chains also tumble at a sufficiently fast rate ($\omega_L \tau < 1$). A rough estimate for the "white" rate is $1.4\ s^{-1}$. The large molecules' tumbling time is roughly $\approx 10^{-7}$ s. This tumbling is responsible for the frequency dependence of T_1.

In phase I the relaxation rate at high fields approximately equals

$$1/T_1 = A + B[\tau/(1 + \omega^2\tau^2) + 4\tau/(1 + 4\omega^2\tau^2)]. \qquad (2)$$

A, the fast diffusion rate, is $\sim 0.4\ s^{-1}$, but may be more if the diffusion has a distribution of correlation times shifted towards longer τ_c (as compared to pure water). The temperature dependence of T_1^{-1} shows that the rate increases by $\sim 0.7\ s^{-1}$ as the temperature is lowered from 293 to 273°K, which can only be the result of slowing down of the fast diffusion of the free state water. It should be noted that for this process

($\omega\tau < 1$) the rate is proportional to τ. If the relaxation rate increases by 0.7 s^{-1}, approximately by a factor of two, it follows that $\tau_d(273)/\tau_d(293) \sim 2$.

In conclusion, tissues in phase I show dispersion of T_1 and of $T_{1\rho}$. The T_1 dispersion is due to the intramolecular (water reorientation; large molecule tumbling) and intermolecular (water diffusion) processes. The dispersion of $T_{1\rho}$ is to a large degree intermolecular (water diffusion) in origin.

The large difference between pure ice and ice in muscle has a twofold origin. Ice crystals in muscle contain ions and other molecules. As a result the water molecule reorientations differ from those in pure ice lattice. In addition, each small ice crystal is contained in a waxy matrix of large molecules and nonfreezable water having the proton T_1 shorter by about two orders of magnitude. For this reason spin flips transmit the information on spin population from the "cold" waxy matrix on the surface of each ice crystal into its interior. Because the size of ice crystals and the conditions on the surface vary, no numerical evaluation of tissue ice T_1 was attempted. We expect little information can be gained from a detailed frequency and temperature variation T_1 study of ice protons in tissues under different cooling rates. It is important, however, to consider the ice proton relaxation in order to prepare properly the experiment on proton relaxation of nonfreezable water and of large molecules; e.g., any effect of ice can be removed by maintaining high enough repetition rates that the ice magnetization (with long T_1) is saturated (Fig. 3). Equivalently, if the FID is studied at times larger than 30 μs, ice magnetization is also left out.

The T_1 anomaly at T_c (Fig. 4), where T_1 drops from ≈ 200 to ≈ 85 ms, can be probed with Eq. 1 under various assumptions. If in phase II only bound water relaxation were observed, then $(T_1^{-1})_{II} = T_{1b}^{-1}$. In such a case, extrapolating $(T_1^{-1})_{II}$ to T_c gives T_{1b}^{-1}, which can be used in Eq. 1 in conjunction with $T_1^{-1}(T_c)_I$ (Fig. 1) to yield $b = 40\%$. This large percentage of bound water appears unlikely, however. If a fraction of the free state water remains liquid in phase II it could be proposed that T_1 anomaly is the result of a change in b, but that the relaxation rate T_{1b} remains unchanged across T_c. By using $T_1^{-1}(T_c)_I$ (with b_I) and $T_1^{-1}(T_c)_{II}$ (with b_{II}), and same T_{1b}^{-1} as well as $(1 - b_I)T_{1f}^{-1} = 0.4\ s^{-1}$ in both equations, it follows that $b_{II} = 2.5\ b_I$. This small increase in b contradicts the fact that almost 90% of the total water freezes. Since the bound state does not freeze as the temperature is lowered across T_c, b_{II} is expected to rise to $\approx 10 b_I$. The free state water in phase II may, however, differ from that in phase I. For example if $(1 - b_{II})T_{1f}^{-1}$ at T_c, extrapolated from phase II, is 2 s^{-1}, and if T_{1b}^{-1} is kept constant in both phases and if $(1 - b_I)\ T_{1f}^{-1}$ is 0.4 s^{-1}, then Eq. 1 requires that b_{II} is equal to 2.1 b_I. This result again does not seem satisfactory. It shows, however, that a variation of T_{1f} has a minor influence on b. One further possibility is to require that $b_{II} = 10 b_I$, and allow $T_{1b}^{-1}(T_c)_{II}$ to be smaller than $T_{1b}^{-1}(T_c)_I$ but keep both weighted free rates equal to 0.4 s^{-1}. Eq. 1 then gives $T_{1b}^{-1}(T_c)_I \sim 4T_{1b}^{-1}(T_c)_{II}$. This result implies that the slow relaxation in bonded state ($\tau\omega_L > 1$) had slowed down at T_c by a factor of four.

In summary, in this heterogeneous system it is not possible to analyze the T_1 anomaly at T_c with Eq. 1 in a unique way. If Eq. 1 is used under the conditions $b_{II} =$

$10b_1$, and if the free rate of 0.4 s^{-1} is left unchanged across T_c, the relaxation rate of bound water is reduced at the transition from phase I to phase II by a factor of four. It should be noted that the above estimates were made on the assumption that bounded water reorients. Identical conclusion would be arrived at if water diffuses (intermolecular) as well as reorients (intramolecular) in the bonded state.

The implication of the above exercise is that water in phase I and the nonfreezable water in phase II may be comparable; e.g., both may obey the rate Eq. 1. If this were the case, the nonfreezable water would consist of a fraction $\sim 20\%$ of slowly reorienting (and diffusing) bound water and of remaining water in a "less bound" state. In this state the diffusion (fast in phase I) is expected to be much slower, however. It is expected that its rate is considerably increased (as compared to 0.4 s^{-1} in phase I) and is frequency dependent. Then, both bound and less bound state water relaxation rates show frequency dependence and their contributions to T_1 may be quite similar.

The observed relaxation extrema in the solid phase (see Table I and Figs. 5–9) can be assigned as follows. The process labeled γ does not change upon deuteration; thus it could only be due to relaxation of end groups like CH_3 and NH_3. The broadness of the two extrema shows that there is a considerable distribution of end group sites. For this reason the activation energy cannot be estimated from the slopes of T_1 or $T_{1\rho}$ vs. the inverse temperature. The process α is not seen in the deuterated sample, thus it can only be the relaxation extremum of the so called nonfreezable water. This mechanism relates to the dispersion of T_1 in the phase I, associated with reorientation and slow diffusion of water in the bound state. The relaxation δ is also due to nonfreezable water (see Table I). It seems plausible that this is the faster motion of reorientation and translational diffusion of nonfreezable water.

In summary, the nonfreezable water undergoes translational and reorientational motions, which show in a T_1 and $T_{1\rho}$ extremum, respectively, in phase II. The large molecules show end groups' effectiveness in two relaxation extrema, as well as a side-chain mobility. This side-chain mobility can be seen as a low-temperature side of a minimum (Fig. 9), which would exist in phase I if the side-chain tumbling did not change across the phase transition T_c. A more quantitative description of molecular motion is possible only by comparing the progressively more deuterated tissues. An experiment along this line is being pursued in our laboratory.

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