

# PROTON NUCLEAR MAGNETIC RESONANCE RELAXATION MEASUREMENTS IN FROG MUSCLE

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**ABSTRACT** Proton nuclear magnetic resonance (NMR) relaxation measurements are reported for frog muscle as a function of temperature and Larmor frequency. Each  $T_{1\rho}$ ,  $T_2$ , and  $T_1$  measurement covered a time domain sufficient to identify the average relaxation time for most intracellular water. Using regression analysis the data were fit with a model where intracellular water molecules are exchanging between a large compartment in which mobility is similar to ordinary water and a small compartment in which motion is restricted. The regression results suggest that: the restricted compartment exhibits a distribution of motions skewed toward that of free water; the residence time of water molecules in the restricted compartment is approximately 1 ms; and, the activation entropy for some water molecules in the restricted compartment is negative.

## INTRODUCTION

Nuclear magnetic resonance (NMR) studies can provide valuable information about the dynamic properties of tissue water. The parameters that can be measured with pulsed NMR techniques include the spin-lattice relaxation time in the laboratory frame ( $T_1$ ) and in the rotating frame ( $T_{1\rho}$ ), the spin-spin relaxation time ( $T_2$ ), and the diffusion constant ( $D$ ).

Early investigators (1, 2) found that  $T_1$  and  $T_2$  for muscle water were much shorter than in ordinary water. The reduced  $T_1$  and  $T_2$  values indicated that the rotational and translational mobility of muscle water was nearly two orders of magnitude lower than ordinary liquid water. However, subsequent measurements of  $D$  showed unequivocally that the average translational mobility of most muscle water is reduced only by a factor of two (3-6).

Today, it is generally believed that NMR relaxation times reflect the reduced mobility of hydrated or bound water (3, 7-9). NMR relaxation times may prove to be a very sensitive tool for studying the dynamic properties of hydrated water if adequate models are developed for their interpretation. Outhred and George (8) found that muscle water  $T_1$  data measured as a function of temperature and Larmor frequency were consistent with a model where a few percent of muscle water had a distribution of mobilities between those of ice and liquid water; in this model, the motional freedom

of the remaining muscle water (~97%) was considered to be similar to ordinary water in agreement with measured diffusion constants.

The  $T_1$  measurements of Outhred and George (8) were most sensitive to the faster motions in hydrated water due to the range of Larmor frequencies used in their study. They did measure  $T_2$  which is sensitive to slower motions and found little dependence of this parameter upon either Larmor frequency or temperature. They did not use  $T_2$  data in their model development.

Koenig and Shillinger (10) have shown that  $T_1$  measurements in protein solutions can provide information about the slower motions of hydrated water if relaxation occurs in magnetic fields approaching zero; facilities for making these measurements are not commonly available. An alternative method of obtaining information about slower motions is to measure spin-lattice relaxation in the rotating frame.  $T_{1\rho}$  measurements in mouse muscle at room temperature have been reported by Thompson et al. (11).

In the present study,  $T_1$ ,  $T_2$ , and  $T_{1\rho}$  have been measured in frog muscle as a function of temperature and Larmor frequency. The range of Larmor frequencies covered in the laboratory frame was not as extensive as in the  $T_1$  measurements of Outhred and George (8). However, Larmor frequencies in the laboratory and rotating frame covered a span of over  $10^4$ . By combining all  $T_{1\rho}$ ,  $T_1$ , and  $T_2$  data in a regression analysis, a new quantitative model of muscle water has been developed.

#### METHODS AND MATERIALS

In liquid water and ice, NMR relaxation occurs via dipolar interactions, mainly intramolecular, between water protons. These interactions are modulated by the motion of the water molecules; this motion is characterized by a correlation time,  $\tau$ , which is a measure of the time required for a molecule to undergo an incremental motion (12-14). Expressions for the  $T_1$ ,  $T_2$ , and  $T_{1\rho}$  relaxation times are, assuming intramolecular interactions only,

$$\frac{1}{T_1} = C \left[ \frac{2\tau}{1 + \omega_0^2 \tau^2} + \frac{8\tau}{1 + 4\omega_0^2 \tau^2} \right], \quad (1)$$

$$\frac{1}{T_2} = C \left[ 3\tau + \frac{5\tau}{1 + \omega_0^2 \tau^2} + \frac{2\tau}{1 + 4\omega_0^2 \tau^2} \right], \quad (2)$$

$$\frac{1}{T_{1\rho}} = C \left[ \frac{3\tau}{1 + 4\omega_1^2 \tau^2} + \frac{5\tau}{1 + \omega_0^2 \tau^2} + \frac{2\tau}{1 + 4\omega_0^2 \tau^2} \right], \quad (3)$$

where:  $\omega_0$  is the NMR Larmor angular frequency in the constant magnetic field,  $H_0$ ;  $\omega_1$  is the Larmor frequency in the rotating frame in the presence of a radio frequency (RF) field,  $H_1$ ; and  $C$  is a constant.

Both  $\omega_0$  and  $\omega_1$  are variable parameters. From Eqs. 1-3, we see that if  $\tau$  is such that  $\omega_0 \tau > 1$ , information about motion can be obtained by studying the  $\omega_0$  dependence of  $T_1$ . The parameter  $\omega_0$  can commonly be varied over a range of about  $10^7$ - $10^9$  rad/s. In pure water,  $\tau$  is so small ( $<10^{-11}$  s) that  $\omega_0 \tau \ll 1$  even at the highest attainable  $\omega_0$  values; this explains (See Eqs. 1-3) why  $T_1 = T_2$  in pure water. These equations also show that for all  $\tau$  values,  $T_2 \leq T_{1\rho} \leq T_1$ .

Additional information about mobility can be obtained by measuring the NMR relaxation time as a function of temperature. A change in temperature produces a change in rotational freedom and a corresponding change in  $\tau$ . For example,  $T_1$  increases with increasing temperature (decreasing  $\tau$ ) if  $\tau$  is in the range such that  $\omega_0\tau < 1$  and decreases with increasing temperature if  $\tau$  is such that  $\omega_0\tau > 1$ . An analogous temperature dependence is expected for  $T_{1\rho}$  for  $\tau$  values giving  $\omega_1\tau < 1$  and  $\omega_1\tau > 1$ , respectively.  $T_{1\rho}$  measurements made with  $\omega_1 \sim 10^5$  to  $10^7$  rad/s can provide information about the longer correlation times that we believe to be characteristic of some intracellular water. In the present study,  $\omega_0$  has been varied over a range from  $125.7 \times 10^6$  rad/s to  $172.8 \times 10^6$  rad/s and  $\omega_1$  over a range of  $3.5 \times 10^4$  rad/s to  $57.9 \times 10^4$  rad/s.

### *The Spectrometer*

A phase coherent spectrometer constructed in our laboratory was employed for all measurements in this study. The sample probe contained a single coil for transmitting and receiving. The recovery time of the RF receiver after a large RF pulse was approximately 40  $\mu$ s. The probe was completely shielded to eliminate RF interference in the magnet regulator, a Magnion model FFC-4 (Magnion, Burlington, Mass.), during the long RF pulses required for rotating frame measurements. The separation, width, and amplitude of the RF pulses were monitored continuously at the coil with a high voltage Tektronix probe (P601SX1000) and a Tektronix oscilloscope (model 454A) (Tektronix, Inc., Beaverton, Ore.).

To avoid problems of nonlinear video detection of the NMR signal, all data were obtained directly with a model 454A Tektronix oscilloscope; i.e., the amplified RF echo and induction decay signals were observed directly without video detection. To insure that the spectrometer was operating properly, relaxation time measurements were routinely made on a standard glycerin sample.

### *Spin-Spin Relaxation Measurements*

Most investigators have reported a single  $T_2$  ( $\sim 45$  ms) for muscle water. However, recent studies over an extended time domain have identified, in addition, a shorter relaxation time ( $< 5$  ms) and a longer relaxation time ( $\sim 200$  ms) (15, 16). The intermediate and short relaxation times have been attributed to intracellular water. The longest relaxation time has been identified with extracellular water.

In the present study,  $T_2$  was obtained with the spin-echo technique using the  $\pi/2$ - $\pi$  pulse sequence (17); the amplitude,  $M(t)$ , of the spin-echo is monitored as a function of  $t$ , the time interval between  $\pi/2$  and  $\pi$  pulses.  $T_2$  and  $M(t)$  are related by the function,

$$M(t) = M(0) \exp(-2t/T_2). \quad (4)$$

The range of  $2t$  in this study extended from approximately 2 ms to 100 ms; this was sufficient to characterize the major intermediate relaxation component in muscle with  $T_2 \sim 45$  ms. A typical spin-echo decay curve is shown in Fig. 1.

### *Spin-Lattice Relaxation in the Rotating Frame*

Measurements were made using the Solomon technique (18); the sample is subjected to a  $\pi/2$  pulse which is followed immediately by a second pulse of amplitude,  $H_1$ , and duration,  $t$ . The second pulse is phase shifted by  $90^\circ$ .  $T_{1\rho}$  is obtained by monitoring the amplitude,  $M(t)$ , of the RF induction decay following the second pulse:

$$M(t) = M(0) \exp(-t/T_{1\rho}). \quad (5)$$

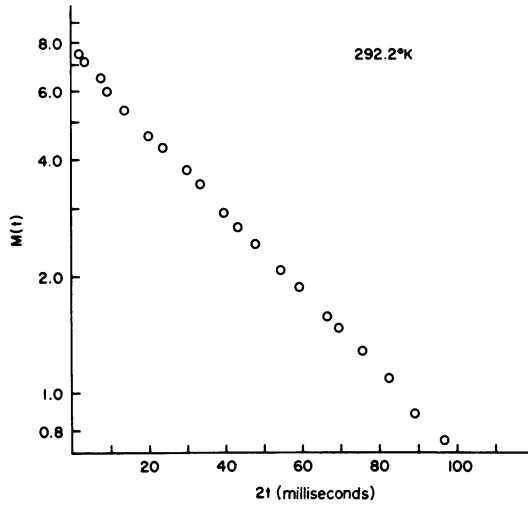


FIGURE 1 Spin echo amplitude  $M(t)$ , plotted against  $2t$  where  $t$  is the time between a  $\pi$  and  $\pi/2$  pulse. The slope of this semilog plot is proportional to  $T_2$ .

The range of  $t$  values in this study was  $\sim 0.1$  ms to 150 ms. A typical decay curve is shown in Fig. 2. There was usually evidence of a short  $T_{1\rho}$  ( $< 5$  ms) representing perhaps 5% of the water which, presumably, corresponds to the small intracellular fraction previously reported from  $T_2$  observations (16). The remaining data could be characterized by a single  $T_{1\rho}$  which we attribute to the large intracellular water fraction determining the  $T_2$  data in Fig. 1. Since  $T_{1\rho} > T_2$  the fraction with  $T_2 \sim 200$  ms attributed to extracellular water (16), could not be observed within our limited measurement time domain.

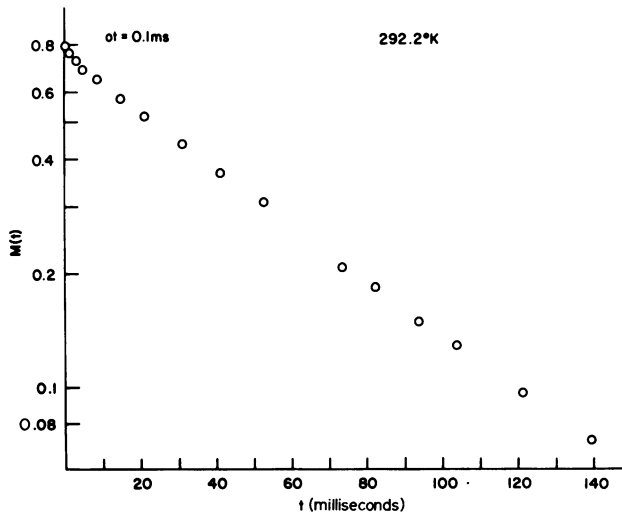


FIGURE 2 Induction signal amplitude,  $M(t)$ , plotted against  $t$ , the length of the preceding pulse. The slope of this semilog plot is proportional to  $T_{1\rho}$ .

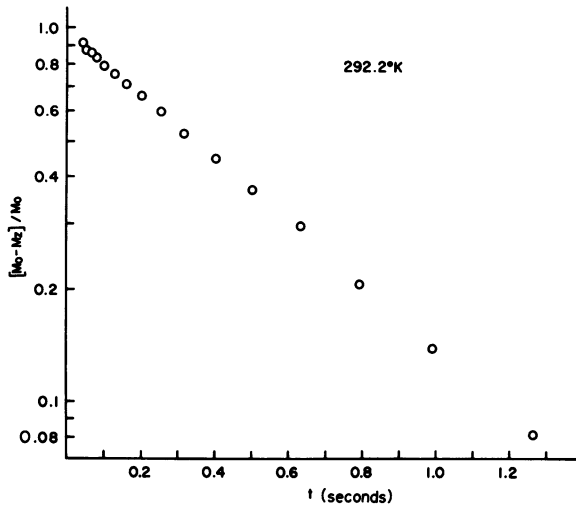


FIGURE 3 Normalized induction signal amplitude,  $[M_0 - M_z]/M_0$  plotted against  $t$ , the time between  $\pi/2$  pulses. The slope of this semilog plot is proportional to  $T_1$ .

### Spin-Lattice Relaxation

$T_1$  measurements were made by monitoring the amplitude of the induction signal,  $M_z$ , following a  $\pi/2$  pulse.  $M_z$  is related to  $T_1$  by the relationship (19),

$$M_z = M_0(1 - \exp(-t/T_1)), \quad (6)$$

where  $t$  is the time between  $\pi/2$  pulses.

The range of  $t$  values in this study was  $\sim 25$  ms to  $\sim 1.2$  s; the lower limit was set by interference from  $\pi/2$ - $\pi/2$  spin-echoes and the upper limit by signal to noise. A typical decay curve is shown in Fig. 3. The data can be characterized by a single exponential which we attribute to the large intracellular fraction of muscle water; i.e., the same fraction identified with the  $T_2$  decay in Fig. 1 and the  $T_{1\rho}$  decay in Fig. 2.

### Sample Preparation and Handling

*Rana pipiens* frogs were obtained from Southwestern Scientific, Tucson, Ariz. They were kept in an aquarium (55°F) without food. After killing the frog by pithing, the gastrocnemius muscle was excised, blotted to remove excess water, inserted into a test tube (12 mm OD), and placed in the NMR sample probe for measurements. NMR measurements were made on frogs kept less than 1 wk after delivery.

Sample temperature was regulated with a Varian Temperature Controller (Varian Associates, Palo Alto, Calif.). Temperatures were monitored with a calibrated YSI needle thermistor immediately before and after each NMR measurement. The uncertainty in sample temperature was less than  $\pm 1/2^\circ\text{C}$  across the sample.

For a given sample at a particular temperature, all NMR measurements ( $T_2$ ,  $T_1$ , and  $T_{1\rho}$  as a function of  $\omega_1$ ) were completed within 2 h after the animal was killed. Each muscle was checked for excitability after the NMR measurements were completed. As an additional check on viability a  $T_2$  measurement was routinely made at the beginning of the NMR measurements and then repeated after  $T_1$  and  $T_{1\rho}$  measurements were completed.

## RESULTS

The majority of the measurements were obtained with a Larmor frequency of 23.3 MHz; these data are plotted in Fig. 4 to show the temperature dependences of  $T_{1\rho}$ ,  $T_2$ , and  $T_1$ . Results for other Larmor frequencies are listed in Table I; the uncertainty in the relaxation times is estimated to be  $\pm 5\%$ . The effect of the slowly relaxing fraction, attributed to extracellular water (16), on this determination of the intracellular relaxation times is not known. However, judging from straightness of the decay curves such corrections would be smaller than the stated uncertainty.

We see that  $T_1$  increases with increasing temperature, indicating, from Eq. 1, motional narrowing and  $\omega_0\tau < 1$  or  $\tau < 10^{-9}$  s. An examination of the  $T_1$  data in Table I and Fig. 4 also shows, in agreement with the previous studies of Outhred and George (8), that  $T_1$  is a function of the Larmor frequency; Eq. 1 indicates that this can only be the case if  $\omega_0\tau > 0.1$ ; i.e.,  $\tau$  must be greater than  $\sim 10^{-10}$  s. Therefore,  $T_1$  seems to be dominated by motions with correlation times in the range  $10^{-9}$  to  $10^{-10}$  s.

Fig. 4 shows that  $T_{1\rho}$  is much smaller than  $T_1$ , is a function of  $\omega_1$ , and increases with temperature. From Eq. 3 and arguments similar to those in the previous paragraph,  $T_{1\rho}$  appears to be determined mainly by motional correlation times ranging

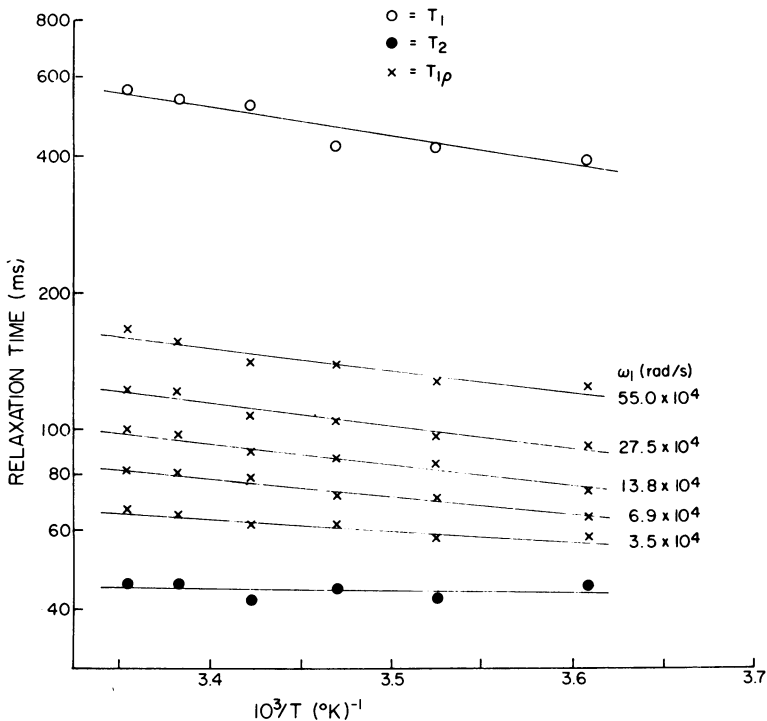


FIGURE 4 Temperature dependence of  $T_{1\rho}$ ,  $T_1$ , and  $T_2$  proton NMR relaxation times of muscle water. All data shown were obtained at  $\omega_0 = 146.4 \times 10^6$  rad/s. The uncertainty in data is estimated to be  $\pm 5\%$ .

TABLE 1\*  
 $T_1$ ,  $T_2$ , AND  $T_{1\rho}$  PROTON NMR RELAXATION MEASUREMENTS  
 ON FROG GASTROCNEMIUS MUSCLE

Temperature	$\omega_1 \times 10^{-4}$	$\omega_0 \times 10^{-6}$	$i \ddagger$	$T_i \S$
$^{\circ}K$	<i>rad/s</i>	<i>rad/s</i>		<i>ms</i>
295.7	0	172.8	2	44
295.7	0	172.8	1	700
295.7	55.0	172.8	3	180
295.7	27.5	172.8	3	137
295.7	13.8	172.8	3	99
295.7	6.9	172.8	3	83
295.7	3.5	172.8	3	72
287.5	0	166.5	2	46
287.5	0	166.5	1	490
287.5	49.4	166.5	3	131
287.5	37.1	166.5	3	114
287.5	24.7	166.5	3	107
287.5	11.3	166.5	3	81
287.5	0	125.7	2	47
287.5	0	125.7	1	390
287.5	57.9	125.7	3	135
287.5	43.4	125.7	3	127
287.5	28.9	125.7	3	112
287.5	14.5	125.7	3	92

\*Data obtained with  $\omega_0$  values other than  $146.4 \times 10^6$  rad/s.

‡The index  $i$  has values of 1, 2, and 3 for  $T_1$ ,  $T_2$ , and  $T_{1\rho}$ , respectively.

§The  $\pm 5\%$  uncertainty in these data results from the uncertainty in the slope of the corresponding relaxation decay curves.

from  $10^{-5}$  s for the smallest  $\omega_1$  to  $10^{-7}$  s for the largest  $\omega_1$ . Together the  $T_{1\rho}$  and  $T_1$  data indicate a range of correlation times in intracellular water extending from  $10^{-5}$  s to  $10^{-10}$  s).

For  $T_1$ , those correlation times shorter than  $1/\omega_0$  would cause  $T_1$  to increase with increasing temperature while those longer than  $1/\omega_0$  would cause  $T_1$  to decrease with increasing temperature. Similarly, with increasing temperature, correlation times shorter than  $1/\omega_1$  and longer than  $1/\omega_1$  would cause  $T_{1\rho}$  to increase and decrease, respectively. Since no minima are evident and both  $T_1$  and  $T_{1\rho}$  increase with increasing temperature, the distribution of correlation times appears to be skewed toward shorter correlation times.

Thompson et al. (11) measured the  $\omega_1$  dependence of  $T_{1\rho}$  in mouse muscle at room temperature in the range  $\omega_1 \sim 10^5$  to  $10^7$  rad/s. Their results showed approximately the same  $\omega_1$  dependence as our data at room temperature. They analyzed their data using an expression of the form,

$$1/T_{1\rho} = R + (A\tau_c/[1 + 4\omega_1^2\tau_c^2]). \quad (7)$$

The second term in this expression resembles the first term in Eq. 3. However, these

authors identified  $\tau_e$  with the exchange correlation time of water protons rather than the rotational correlation time of water molecules. The constant  $A$  is defined only as a measure of the "interaction which is being modulated by the exchange"; the nature of this interaction is not identified. The constant  $R$  is identified with the "proton relaxation rate in the absence of exchange" which must be the dipolar relaxation rate given by Eq. 3. These authors treat  $R$  as a constant neglecting its  $\omega_1$  dependence which is appreciable in their experiment for rotational correlation times in the range of  $10^{-5}$  s to  $10^{-7}$  s. Interestingly, a  $\tau_e = 5 \times 10^{-6}$  s was obtained in their analysis.

The proton exchange correlation time in liquid water is approximately  $10^{-3}$  s (20). For water of hydration it appears that the proton exchange correlation time may be decreased by as much as an order of magnitude (Woessner, D. E., unpublished results). However, an exchange correlation time of  $5 \times 10^{-6}$  s for muscle water clearly seems out of the range of expectations. We believe that the  $\tau_e$  in Eq. 7 can be more reasonably identified with the rotational correlation time of water molecules.

### ANALYSIS

We have chosen a two-compartment model for analysis; it assumes that the data may be represented by a large compartment of free water with a correlation time,  $\tau_w$ , and a small compartment with restricted motions and a distribution of correlation times. The model also assumes that water molecules are exchanging between these two compartments in a time short compared with the NMR observation time. With these assumptions, the calculated relaxation rate (the reciprocal of the relaxation time) is given by a weighted average of the rates in each compartment (16):

$$(1/T_i)_{\text{cal}} = (F_w/T_{iw}) + ((1 - F_w)/[T_{iR} + R_i]). \quad (8)$$

The index  $i$  has values of 1, 2, and 3 for  $T_1$ ,  $T_2$ , and  $T_{1\rho}$ , respectively.  $T_{iw}$  and  $T_{iR}$  represent the calculated relaxation times in the free and restricted compartments, respectively.  $R_i$  is the residence time of a water molecule in the restricted compartment.  $F_w$  and  $(1 - F_w)$  represent the fractions of water in free and restricted compartments, respectively.

Since  $T_{iw}$  is characterized by a single correlation time ( $\tau_w$ ) it can be calculated directly from Eqs. 1, 2, or 3. The relaxation rate in the restricted compartment is calculated assuming a distribution of  $M$  discrete correlation times:

$$\frac{1}{T_{iR}} = \sum_{j=1}^M \frac{f(j)}{T_i(\tau_j)}. \quad (9)$$

Again the index  $i$  takes on values of 1, 2, and 3 and the  $T_i(\tau_j)$ 's are calculated from Eqs. 1-3. The distribution weighting function,  $f(j)$ , is taken as,

$$f(j) = \exp(jB_1 + j^2B_2) / \sum_{j=1}^M \exp(jB_1 + j^2B_2). \quad (10)$$



The choice of this discrete distribution greatly simplifies computations and should provide an approximate picture of any continuous distribution.

The temperature dependence of motion in both compartments is assumed to depend upon the free energy of activation,  $\Delta G^*$ , in the same manner as the dielectric relaxation time in liquid water (21):

$$\tau = (h/3.7kT) \exp(\Delta G^*/RT). \quad (11)$$

In this expression,  $h$  is Planck's constant,  $k$  is Boltzmann's constant,  $R$  is the ideal gas constant, and  $T$  is the absolute temperature. The factor of 3.7 comes from the experimental observation that the NMR correlation time in free water ( $\tau_w$ ) is smaller than the dielectric relaxation time in free water by a factor of 3.7 (21).  $\Delta G_w^*$  is calculated from the activation enthalpy ( $\Delta H_w^*$ ) and entropy ( $\Delta S_w^*$ ) obtained from dielectric relaxation measurements in free water (21) ( $\Delta H_w^* = 4.2$  kcal/mol,  $\Delta S_w^* = 6.1$  cal/mol-deg).

We assume that each discrete correlation time,  $\tau_j$ , in the restricted compartment is characterized by an activation free energy,  $\Delta G_j^*$ . The free energies are calculated from an expansion in temperature about the midrange of our observations (288.2°K):

$$\Delta G_j^* = (j - B_3)B_4 + (T - 288.2)(B_5 + (j - B_3)B_6). \quad (12)$$

Note that the activation enthalpy,  $\Delta H_j^*$ , and activation entropy,  $\Delta S_j^*$ , can be defined as follows,

$$\begin{aligned} \Delta H_j^* &= \Delta G_j^* \quad (\text{evaluated at } T = 0) \\ -\Delta S_j^* &= (\Delta G_j^* - \Delta H_j^*)/T \quad (T \neq 0). \end{aligned} \quad (13)$$

A similar expansion is used to estimate the temperature dependence of the residence time of a water molecule in the restricted compartment:

$$R_i = B_7 + B_8(T - 288.2). \quad (14)$$

The parameters  $B_1$  through  $B_8$  can be calculated by a least squares fit to the data. The computer program we have used adjusts nine parameters ( $B_1$  through  $B_8$  plus  $F_w$ ) to minimize the sum of square errors (SSE) from  $N$  observations.

$$\text{SSE} = \sum_{i=1}^{N=61} [\log(1/T_i)_{\text{obs}} - \log(1/T_i)_{\text{cal}}]^2. \quad (15)$$

The 61 observations represent the relaxation data points in Fig. 4 and Table I. The iterative algorithm we have used is a blend of the gradient method (22), the method of Hartley (23), and the Marquardt algorithm (24).

### Estimating the Constant $C$

Before beginning the regression procedure, the constant  $C$  in Eqs. 1-3 had to be approximated. For intramolecular interactions this parameter can be defined exactly (13).

$$C = (3/4)\gamma^4\hbar^2/20r^6, \quad (16)$$

where  $\gamma$  is the nuclear gyromagnetic ratio,  $\hbar$  is Planck's constant divided by  $2\pi$ , and  $r$  is the distance between protons on a water molecule. Using a value of  $r = 1.58 \text{ \AA}$ , the intramolecular  $C$  is calculated to be  $0.549 \times 10^{10} \text{ s}^{-2}$ .

Unlike intramolecular interactions, intermolecular interactions are dominated by the translation of water molecules (13). Eisenberg and Kauzmann (25) have shown that rotation and translation of water molecules in water are strongly coupled; following Resing (26) and Outhred and George (8), we will make the approximation that a single correlation time can be used to characterize both motions. In this case,  $3C$  is equal to the rigid lattice second moment,  $\sigma$ . From the measured value of  $\sigma$ ,  $2.4 \times 10^{10} \text{ s}^{-2}$  (14, 27),  $C$  becomes  $\sim 0.8 \times 10^{10} \text{ s}^{-2}$ .

### Regression Results

Initial regressions were carried out with  $C$  fixed at the intramolecular value,  $0.549 \times 10^{10} \text{ s}^{-2}$ . Regressions were run using different numbers of components in the distribution of correlation times for the restricted compartment. The best fit occurred with five components ( $M = 5$ ); this fit was significantly better than either four or six components. The standard deviation for this regression ( $M = 5$ ) fit was 0.051; this

TABLE II  
REGRESSION RESULTS FOR  $M = 5$

Parameter	Estimate	SE
$F_w$	0.972	0.001
$B_1$	-2.16	0.16
$B_2$	0.144	0.035
$B_3$	-3.37	0.13
$B_4$	$13.32 \times 10^2$	29.0
$B_5$	-86.0	16.0
$B_6$	16.6	3.1
$B_7$	$0.80 \times 10^{-3}$	$0.14 \times 10^{-3}$
$B_8$	$0.70 \times 10^{-5}$	$0.71 \times 10^{-5}$

This table shows the regression results when the restricted compartment contained five discrete correlation times.  $B_4$  is in calories per mole.  $B_5$  and  $B_6$  are in calories per mole-degree.  $B_7$  is in seconds and  $B_8$  in seconds per degree centigrade. All other parameters are dimensionless. SE represents the standard error of each parameter. The standard deviation for this regression fit was 0.051.

corresponds to our expectations based on the uncertainty in the experimental observations. The regression results for the five component distribution are listed in Table II.

Other regressions were run with  $C$  increased to  $0.8 \times 10^{10} \text{ s}^{-2}$ . The results were similar to those for the smaller  $C$  with only minor changes in the estimates of parameters.

## DISCUSSION

From Table II, we find that our model predicts that the free compartment contains 97% of the water and the restricted compartment contains 3%. The parameter  $B_7$  indicates that water molecules (at 288.2°K) spend approximately 0.8 ms in the restricted compartment before returning to the free compartment. At 288.2°K, the calculated values of  $T_1$ ,  $T_2$ , and  $T_{1\rho}(\omega_1 = 55 \times 10^4 \text{ s}^{-2})$  in the restricted compartment are 13 ms, 0.4 ms, and 0.9 ms, respectively. The  $T_1$ ,  $T_2$ , and  $T_{1\rho}$  relaxation times in the free compartment are of the order of several seconds. We see that relaxation in the restricted compartment can have a dramatic effect on the observed relaxation time even though this compartment is small compared with the free compartment.

The averaging of relaxation in the two compartments can only occur if observation times are long compared to the residence time in the restricted compartment; i.e., long compared with  $R_1$ . We would predict that measurements made at times less than or equal to  $R_1$  would allow the direct observation of relaxation in the restricted com-

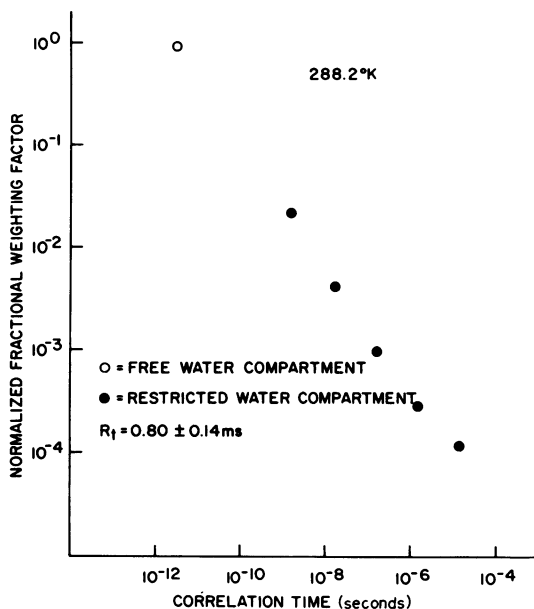


FIGURE 5 Fractional weighting factors are plotted against the corresponding correlation time for the five-component distribution for the restricted compartment.

partment. Although not well defined, the short intracellular  $T_2$  recently observed in muscle was approximated to be 0.42 ms (16). We believe that this  $T_2$  can be identified, at least in part, with the restricted compartment in our model.

#### *Distribution of Correlation Times*

Fig. 5 shows the normalized fractional distribution of correlation times calculated from the regression results in Table II. As expected, the distribution is strongly skewed toward the free water correlation time. It has the same general shape as the truncated continuous distribution reported by Outhred and George (8); Walter and Hope (28) were the first to predict a skewed distribution.

#### *Residence Time*

The residence time  $R_i$  at 288.2°K is equal to parameter  $B_7 = 0.80 \pm 0.14$  ms. The temperature dependence of  $R_i$  (given by parameter  $B_8$ ) is not well defined; however, over the temperature spread of our data the uncertainty in  $B_7$  and  $B_8$  indicates that  $R_i$  varies between 0.5 ms and 1.5 ms. Since  $R_i$  is the same order of magnitude as  $T_2$  and  $T_{1\rho}$  in the restricted compartment, we see from Eq. 7 that it contributes significantly to the observed average  $T_2$  and  $T_{1\rho}$  relaxation times. The  $R_i$  contribution is greatest for  $T_2$ , decreases for  $T_{1\rho}$  as  $\omega_1$  is increased, and is negligible for  $T_1$ .

The decision to assign a single residence time for the restricted compartment was made to facilitate computations. Since a distribution of residence times seems more plausible physically, we might consider the calculated value as an average. The uncertainty in the temperature dependence of  $R_i$  may indicate the need for a distribution of residence times.

#### *Activation Parameters*

The thermodynamic activation parameters,  $\Delta H^*$  and  $\Delta S^*$  for the distribution have been calculated from the regression results in Table II and plotted in Fig. 6. When calculating the free energy of activation we find that  $\Delta G_j^*$  varies linearly from the free water value of 2.4 kcal/mol to approximately 11.5 kcal/mol for the distribution component with the longest correlation time  $\sim 10^{-5}$  s. The activation energy for rotational and translational motions in ice is  $\sim 13$  kcal/mol with a correlation time  $\sim 10^{-5}$  s (29). It might seem reasonable, therefore, to think of the longer correlation times in our distribution as being ice-like. However, the difference between the  $\Delta G^*$  of free ice and free water is due mainly to an increase in  $\Delta H^*$  (29) while in our distribution it is due entirely to a decrease in  $\Delta S^*$ .

No independent data are available on the entropy of activation of motionally restricted intracellular muscle water. However, some information is available on other systems. For example, Harvey and Hoekstra (30) have reported dielectric relaxation measurements for water adsorbed on lysozyme. Their data indicate an average entropy of activation of  $-27.3$  cal/mol-deg for the first hydration layer. These authors attribute the depressed freezing point of this water ( $\sim -80^\circ\text{C}$ ) to this decrease in  $\Delta S^*$ ; a small fraction of muscle water also exhibits a greatly depressed freezing point

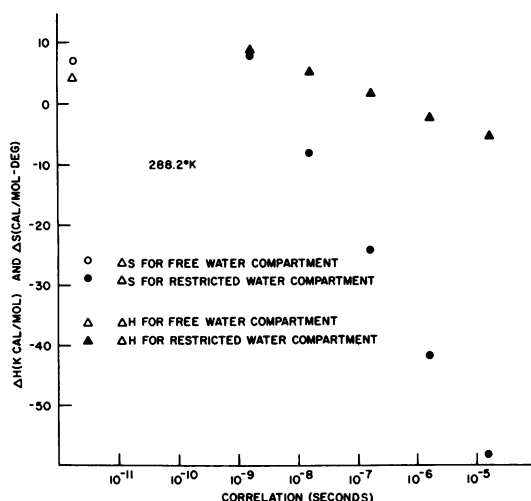


FIGURE 6 Activation enthalpy and entropy for free and restricted compartments are plotted against the correlation time obtained for each component of the distribution.

( $\sim -80^{\circ}\text{C}$ ) which can not be attributed to either eutectic depression or supercooling (15, 31).

Without intermolecular hydrogen bonding,  $\text{H}_2\text{O}$  would be expected to freeze at about  $-90^{\circ}\text{C}$  (32); under these conditions water molecules are said to be *unassociated*. The greatly depressed freezing point of water adsorbed on alumina and silica gel has been attributed to unassociated water molecules (33); the water adsorbed on silica appears to be more ordered than bulk water (lower entropy) and that adsorbed on alumina less ordered.

In another NMR study, Kuntz and Brassfield (34) found a negative activation entropy ( $-10$  cal/mol-deg) for water bound to bovine serum albumin. They made no attempt to assign any physical significance to their observation.

In a recent study of dielectric relaxation of water in gelatine solutions, Masszi (35) reported evidence of increased dielectric relaxation times for water closely associated with the protein. He attributes this not to an increase in  $\Delta H^*$  (stronger hydrogen bonding) but, rather, to a decrease in the  $\Delta S^*$  activation entropy.

## SUMMARY

$T_1$ ,  $T_2$ , and  $T_{1\rho}$  relaxation times have been measured in frog muscle as a function of temperature and Larmor frequency. The range of Larmor frequencies extended from  $3.5 \times 10^4$  rad/s in the rotating frame to  $1.73 \times 10^8$  rad/s in the laboratory frame. Assuming that the relaxation mechanisms are due to dipolar interactions, the data analysis clearly indicates a distribution of correlation times for muscle water ranging from  $\sim 10^{-5}$  to  $\sim 10^{-11}$  s and skewed toward the latter value.

All relaxation data (61 values) have been simultaneously fit with a model which as-

sumes that water molecules are exchanging between two compartments, one with unrestricted motions similar to ordinary water, and one with a distribution of restricted motions. Predictions of the model are consistent with available experimental evidence concerning the dynamic properties of muscle water. In particular, the  $R_1$  and  $T_2$  parameters calculated for the restricted compartment are in agreement with recent measurements over an extended time domain (16).

The opinions in this paper are those of the authors and do not necessarily reflect those of the Navy Department or the naval service. The animals used in this study were handled in accordance with the provisions of Public Law 89-544 as amended by Public Law 91-579, the "Animal Welfare Act of 1970," and the principles outlined in the "Guide for the Care and Use of Laboratory Animals," U.S. Department of Health, Education, and Welfare Publication No. (NIH) 73-23.

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