SELF DIFFUSION OF WATER IN FROG MUSCLE

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ABSTRACT Self diffusion of cell water has been measured at diffusion times ranging from 0.3 ms to 2.4 s for three muscle types of *Rana pipiens*, using various magnetic field gradient nuclear magnetic resonance methods. Intracellular diffusion coefficients and membrane permeabilities are calculated with the aid of previous theoretical results for regularly spaced permeable planar barriers. The intracellular diffusion coefficient is 1.6×10^{-5} cm^2/s, in approximate agreement with other literature values for skeletal muscles. The outer membrane permeabilities are estimated at 0.01 cm/s for two of the muscle types, and much higher for the other one.

INTRODUCTION

The mobility of molecules inside living cells is a measure of the viscosity of the medium there. Mobility may be measured on various distance scales corresponding to the time scale of the particular measurement method.

Rotational and translational modes of motion covering distances of a few angstroms may be observed by nuclear magnetic resonance (NMR) measurements of spin-spin (T_2) and spin-lattice (T_1 and $T_{1\rho}$) relaxation times (1, 2). When more than one type of motion contributes to the spin relaxation or when more than one phase is present (e.g., an absorbed phase), the resolution into the various rotational, diffusional, and exchange components may require measurements over a wide range of experimental parameters (temperature, radio frequency, isotopic dilution) and a complicated analysis of the data in terms of the various motion and exchange rates.

By contrast, a direct unambiguous measurement of translational diffusion over longer distances (typically 1–100 μm) may be made using magnetic field gradient NMR methods (3, 4). We report here measurements of diffusion coefficients of water in three different types of frog muscle cells. A variety of magnetic field gradient techniques were used so as to cover an unusually wide range of diffusion times. The time dependence of the diffusion coefficients is analyzed to obtain the intracellular diffusion coefficients and estimates of the permeability of the cell membranes. A survey of literature results of diffusion measurements within other muscle cells is presented.

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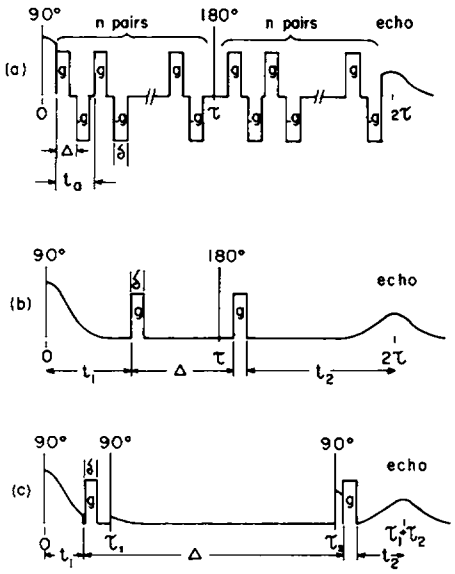

FIGURE 1 Radio frequency (90°, 180°) and field gradient (g, $-g$) pulse sequences suitable for diffusion measurements at (a) short (6), (b) intermediate (4), and (c) long (5) diffusion times, respectively.

THEORY

Method

A full description of magnetic field gradient NMR methods for diffusion measurements is found in the original literature on the subject (3–6). Briefly, one to a few cubic centimeters of sample is contained in a small glass tube (a 4.2-mm i.d. in our case) and placed in a high magnetic field. Strong radio frequency pulses at the resonance frequency tilt the static nuclear magnetization out of alignment with the main field, H_o. The resulting precession about H_o gives rise to an observable signal which decays in time because of various processes. The rate of signal decay is increased if the magnetic field is inhomogeneous and random diffusion of the spins is occurring. The most useful experimental procedure is to artificially create an inhomogeneity of the magnetic field in the form of pulses of a uniform field gradient.

Various sequences of pulses of the radio frequency (rf) and of the magnetic field gradient have been devised, each best suited to a particular range of diffusion times. The sequences used in the present experiments are illustrated in Fig. 1. The sequence of Fig. 1 *b* is in common use for this type of measurement, whereas the sequences of Figs. 1 *a* and 1 *c*, are useful for short and long diffusion times, respectively, and have been rarely used until now. They have in common the application of one or more pairs of gradient pulses, of intensity g, duration δ, and interval Δ (start to start). The time of diffusion is defined by the spacing of the gradient pulses, and is given by $t_d = \Delta - \delta/3$. The diffusional attenuation of the signal, measured at the time of the echo, is given by $\ln R = n\gamma^2 D\delta^2 g^2 (\Delta - \delta/3)$, where R is the ratio of echo amplitudes in the presence and absence of the field gradient, n is the number of pairs of gradient pulses, γ is the nuclear gyromagnetic ratio, and D is the diffusion coefficient.

The T_2 and T_1 relaxation also cause an attenuation of the echo, compared to the signal which is present immediately after the first 90°-rf pulse. The amount of this attenuation is governed solely by τ or by τ_1 and τ_2, which are kept constant for any given measurement of R. Because T_1 and T_2 generally differ for differing groups of spins, it is frequently possible to selectively enhance or eliminate certain groups by proper choice of τ. The proton signals from rigid structures or molecules are easily eliminated by choosing $\tau > 1$ ms.

Restricted Diffusion

On a microscopic level diffusional behavior may be complicated if the local rate of diffusion varies from one region to another within the medium, or if there are barriers to the diffusion. Where the time required to cross less viscous subregions falls within the range of accessible diffusion times, the measured D will be a function of the diffusion time, i.e., $D = D(t_d)$.

In discussing the experimental results, we will assume a diffusing medium having a single local diffusion coefficient D_o, in which are placed barriers (i.e., membranes) of relatively small volume. If diffusion is observed only over a very short time, few of the molecules can contact the barriers, and their motion is primarily determined by the viscosity of the medium between the barriers. Extrapolating to zero diffusion time we obtain D_o. As the observation time is extended, more of the molecules contact the barriers; they move relatively less than they would have in the absence of barriers, and the apparent D decreases. As diffusion time becomes long compared to the interval between encounters with the barriers, the apparent D approaches a constant value, D_∞, which is finite if the barriers are permeable, or zero if the barriers are impermeable.

The value of D at finite times is a complicated function of barrier permeability, geometry, and diffusion time. We will analyze our data using theoretical results derived for the case of equally spaced, parallel planar barriers (7), because to our knowledge that is the only geometry for which a complete theoretical derivation exists, valid at intermediate values of the diffusion time, and for arbitrary barrier permeability.

Typical numerical results showing the theoretical variation of diffusion coefficient with diffusion time are presented in Fig. 2. In the limit of zero time, D/D_o approaches unity, as expected. At very long times, it is verified numerically that D/D_o approaches values which may be calculated from $1/D_\infty = 1/D_o + 1/ap$, an expression readily derived for the case of steady-state diffusion in this geometry. Here a is the barrier spacing, and p is the barrier permeability.

Thus, if measurements extend well enough into the horizontal regions of the curves we can readily estimate D_o, D_∞, and the product ap. If either the permeability or spacing is known independently, we can calculate the other; otherwise we make use of the intermediate portions of the curves. For convenience here we use the locus of points (not explicitly presented) where D reaches its average value,

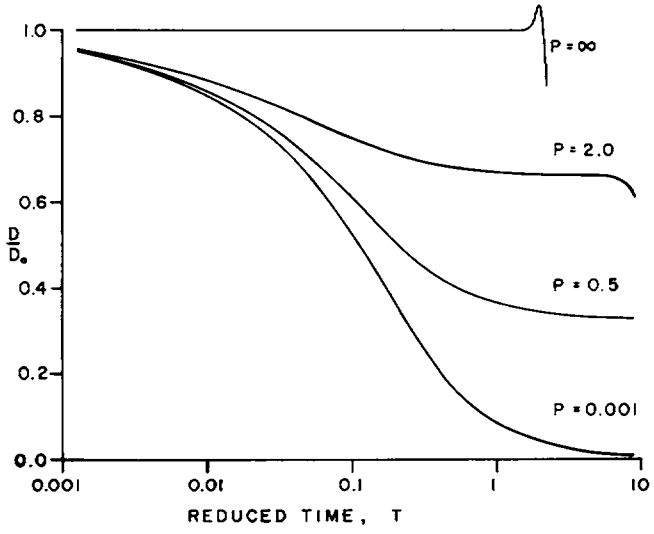

FIGURE 2 Relative apparent diffusion coefficient, D/D_o, vs. reduced diffusion time, $T = D_o\, t/a^2$, for a medium partitioned by parallel planar barriers of spacing a, and reduced permeability $P = ap/D_o$ (taken from reference 7).

$(D_o + D_\infty)/2$. With an experimental value of $P = ap/D_o$ we select the appropriate curve from the family of theoretical curves represented in Fig. 2, and read off $t_{1/2}$, corresponding to D (average). From the experimental plot of D vs. t_d, we estimate that t_d corresponding to D (average) and immediately calculate a, and then p. Note that the "barrier" between tightly packed adjacent cells consists of two membranes, each of permeability twice that for the total barrier.

EXPERIMENTAL

NMR Apparatus and Methods

A Varian HA-60 system (Varian Associates, Palo Alto, Calif.) provided the DC magnetic field, H_o, and also operated a frequency modulated external field-frequency lock at the fluorine resonance frequency. The proton and fluorine resonance frequencies were derived from a Hewlett-Packard frequency synthesizer (Hewlett-Packard Co., Palo Alto, Calif.). The delay and duration of the rf pulses were digitally timed by a Nicolet 293 pulse programmer (Nicolet Instrument Corp., Madison, Wis.).

The diode detector was calibrated to ~ 50 db below saturation by feeding an attenuated continuous wave rf signal into the probe. A linear response was assumed for the few points below this range. Weak NMR signals were time averaged by means of a Nicolet 1080. At most, 32 repetitions were counted.

A switch for gating field gradient pulses of either sign was constructed. The design was similar to that of Gross and Kosfeld (6) except that a modification was made to allow equalization of the current integrals of the positive and negative pulses. This was done so that the time interval within a gradient pulse pair would accurately represent the diffusion time.

Trains of field gradient pulses of alternating sign (Fig. 1 *a*) were used for measurements at the shortest diffusion times, generally 0.3–5 ms. A more conventional two-pulse sequence, as in Fig. 1 *b*, was used for intermediate times, 2–40 ms. A stimulated echo sequence (5) with pulsed or steady applied field gradient was used for diffusion times of 30 ms–2.4 s.

The duration of the gradient pulses was kept constant at 0.4 ms. The intensity was varied up to ~ 500 G/cm. The applied cw gradient, when used alone, was varied up to 1.5 G/cm. The gradient was calibrated by careful measurement of echo widths, generally at half amplitude. Using the calibrated value of the gradient, the D of pure water was found to be 2.3×10^{-5} cm^2/s at 27 ± 2°C, in good agreement with the value 2.41×10^{-5} cm^2/s at this temperature obtained in careful recent measurements by Mills (8).

T_1 were measured by means of a 180°–90°- or a 90°–90°–90°-rf sequence, or both. The probe temperature was 27 ± 1°C for all samples.

Sample Handling

Measurements were made over a 2-d period. At the beginning of each day an adult frog, *Rana pipiens*, was pithed, and some of the muscles dissected intact, being kept moist with a frog Ringer solution.

For the first day's measurements the muscles were immediately pulled into a 4.2-mm i.d. glass NMR tube by means of a thread tied onto the end. Bulk liquid which squeezed into the region at the lower end was blotted off, and both ends of the tube were plugged. The tubes were kept on ice except during the diffusion measurements, which were at a probe temperature of 27°C. One sample of each of three muscle types was prepared—sartorius (sample 1), peroneus (No. 2), and semitendinosus (No. 3)—and measured in that order.

There were a few nonideal aspects of two of these samples. The sartorius muscle did not completely fill the tube even at its middle. This caused difficulties in the measurements as a result of local field gradients. In the case of the peroneus sample, a few fibers were removed to allow it to fit into the tube. Thus, a small neighboring portion of that muscle was dead at the start of the experiment.

Measurements required 1–3 h for each sample. Tests of response to an electrical stimulus were performed immediately upon completion. Sample 1 contracted locally after removal from the tube, and contracted along its entire length after a few minutes in saline solution. Sample 2, after measurement,

was capable only of local contraction even after several minutes in saline solution. Sample 3 was not tested.

For the second day's measurements the dissected muscles were allowed to sit in an ice-cold saline solution except during NMR measurements. Two samples were studied, one pair of sartorius (No. 4) and one pair of semitendinosus muscles (No. 5), with centers staggered so as to entirely fill the NMR tube for some length without excessive squeezing of the center of the muscles. After a maximum of 40 min (usually 30 min) of measurements at probe temperature, the muscles were removed from the tube, allowed to sit 5 min in the saline solution, then in some cases, reloaded into the tube for more measurements. All measurements were completed 3 h after the dissection.

Samples 4 and 5 remained responsive to a small electrical signal throughout the tests. Each time they were removed from the tube they contracted along the entire length upon application of a small voltage at one location, without prior soaking in saline solution. However, by the end of the 3-h measuring time the contractions were perceptibly weaker. The maintenance of viability was especially desired for these samples because they were used for measurements at the longest diffusion times, to detect the restrictive effects of the cell membranes.

RESULTS AND DISCUSSION

T_1 and T_2 relaxation measurements over a factor of 4 in signal strength, and gradient-attenuated signal strengths over a factor of 10 gave no indication of more than a single diffusing species in our samples. However the usual experimental scatter over this range can mask the existence of major components with T's or D's differing by as much as a factor of 3 or minor components with larger differences in T or D. Hazlewood et al. (9) have in fact resolved minor fractions (8 and 10%) of protons with markedly different relaxation times in skeletal muscle of rats by careful T_2 relaxation measurements over three decades of signal attenuation. We accept a similar possibility for our samples, and believe that our relaxation and diffusion measurements are averages over all protons with T_2 long enough to contribute to the signal at τ or τ_1 values of 5–15 ms. These different spin populations would be expected to consist almost entirely of water, because T_2 of the protons in the protein are short compared to the τ values used.

All of the relaxation measurements (Table I) gave the result that T_1 (cellular) $<<T_1$ (pure water) and $T_2 << T_1$. This is typical for cell systems. We accept the common explanation that

TABLE I

SPIN RELAXATION TIMES, DIFFUSION CONSTANTS, AND MEMBRANE PERMEABILITIES IN FROG MUSCLE

	Relaxation times		Measured or extrapolated limiting D's		Reduced permeability $P = ap/D_o$	Time of average D	Spacing a	Permeability, p
	T_2	T_1	D_o	D_∞				
	ms		$\times 10^5$ cm^2/s			*ms*	*μm*	*cm/s*
1 Sartorius		1,400	1.63		1.40	40	43	0.01
4 Sartorius	45*	1,000		0.95				
2 Peroneus	52	1,100	1.76	≥1.2	2.15	33	50	<0.015
3 Semitendinosus	55	1,100	1.74		5.0	12	40	0.04
5 Semitendinosus		1,400		1.45				

*The same result within an uncertainty of ±5% was obtained using a 90°–90° or a 90°–180°-rf pulse sequence.

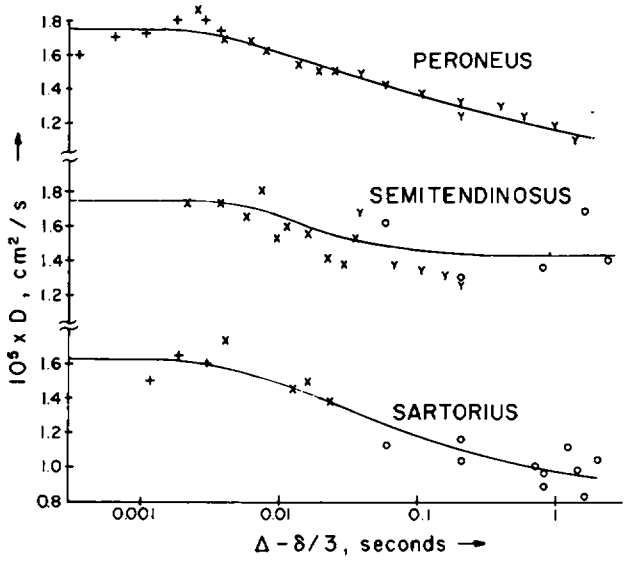

FIGURE 3 D vs. diffusion time in muscle of *Rana pipiens*. Each point is an average of values at two-five field gradient strengths. The smooth curves were drawn by eye.

Symbols	Samples	Method
+	1,2	90°–180°, alternating sign gradient pulses
×	1,2,3	90°–180°, conventional gradient pulses
Y	2,3	90°–90°–90°, conventional gradient pulses, two different τ_1
O	4,5	90°–90°–90°, steady gradient

this is caused by the existence of a major portion of water having motional properties not much different than those of bulk water, and a minor (probably inhomogeneous) portion which has very slow motion (10). Shporer and Civan (11) have shown in a recent review that the available diffusion, relaxation, and line-splitting data on the nuclides ^1H, ^2D, and ^{17}O in the water in biological samples are inconsistent with any simple model of the state of the slower moving fraction.

All of the diffusion results are presented in Fig. 3 as plots of the apparent (time-dependent) D vs. experimental diffusion time. There is a general decrease of D with increase in diffusion time, except at the shortest times where at least for the peroneus, and possibly also for the sartorius and semitendinosus samples the D appears to be constant, to within an uncertainty of perhaps ± 15%. Thus diffusion distances at $t_d \approx 1$ ms are much shorter than the spacing which causes the observed decrease at longer times.

The possibility still exists of additional barriers so closely spaced that the variability of D which they produce occurs at diffusion times shorter than the range covered in these experiments. We will calculate the tightest, most widely spaced barriers which could have escaped detection. For this calculation we take our D at shortest times as D_∞, and assume a D_o equal to that of pure water. From $1 + 1/P = D_o/D_\infty \lesssim 2.3/1.7$ we get $P \gtrsim 3$.

For barriers of this permeability, and spaced widely enough such that $T \lesssim 0.3$ within our

range of measurements, we would expect to have observed a strong time dependence of the D, judging from Fig. 2. Therefore from $T = D_o t/a^2$ we calculate $a < 3$ μm and/or $P >> 3$, $p > 0.2$ cm/s for a set of barriers which would have escaped the observations.

The variability of D's observed within the range of measurements could have been caused by several sets of barriers. However the measurements are not precise enough, nor does the theory of parallel planes accurately enough represent the actual geometry to justify attempting to resolve more than one set of barriers in this range. We therefore ascribe the entire effect to a single barrier type and show that the calculated spacing is reasonably close to the average distance across the cell, so that the observed barrier(s) are well enough represented as being caused by the outer cell membrane.

The calculation is performed as indicated in the theory section. Intermediate and final results are presented in Table I. Calculations with reasonable sets of initial parameters show that the calculated permeabilities are accurate to within a factor of 2, except for the semitendinosus, where the value should only be taken as a lower limit.

Part of the uncertainty is in the estimation of D_∞. The measured values of D did not become constant in spite of the unusually long diffusion times of several seconds. The uncertainty in the calculation of p is increased for the sartorius and semitendinosus samples by the assumption that the samples from two different frogs have the same physical parameters. The calculations for semitendinosus are particularly uncertain due to experimental scatter, which is large compared to the overall change in D with diffusion time.

An additional uncertainty in the calculations arises from the fact that for the ideal system of parallel planes, which we are using as a model for analysis of the data, the measured D is predicted to be a function of the gradient used to make the measurement ($\bar{7}$), as well as of the system parameters. We did not include this effect, but only used the limiting relation at low field gradient. If this effect holds for our muscle cell system, the biggest change would be at the long diffusion times, where the estimated D_∞/D_o could be too low by as much as 20% and the estimated value of P low by as much as a factor of 2.

The present D's of water in frog muscle are compared in Table II with values for other types of muscle obtained by magnetic field gradient methods. The first column gives the type of sample studied and information about sample condition and orientation. The column labeled "diffusant" names the substance, usually protonated water, whose diffusion was observed. The column "D_o/D_w" gives the ratio of the true intracellular D to the D of the same substance in a reference bulk aqueous solution of the same concentration. For instance, where the diffusant is protonated water, the reference medium is pure water. Where the diffusant is tritiated water (HTO), the reference system is a solution of HTO in protonated water. Thus, the ratio D_o/D_w measures the effect of other dissolved substances and of intracellular structures (if unresolved by variation of the diffusion time) on the motion of the diffusant.

The tracer measurements in muscle cells required minutes or hours, but because the direction was parallel to the long axis of the fiber the effect of the finite cell dimensions was still small, and the results are essentially equivalent to NMR measurements at zero diffusion time.

The range of apparent D's and the corresponding range of experimental t_d are given in the next two columns, where $t_d = \Delta - \delta/3$ or $2\tau/3$ for pulsed or steady field gradient experiments, respectively. Note that in nearly all cases where measurements were performed over a range

TABLE II
COMPARISON WITH LITERATURE DIFFUSION COEFFICIENTS

Sample	Diffusant	D_o/D_w	D/D_w	t_d	Reference
Frog, *Rana pipiens*	Water			*ms*	
Peroneus‡,§		0.76	0.76–0.50	0.4–1,400	*
Semitendinosus‡,§		0.76	0.76–0.64	2–2,400	*
Sartorius‡,§		0.72	0.72–0.43	1–2,000	*
Frog, *Rana esculenta*, Gastrocnemius	Water	0.65			14
Frog, *Rana pipiens* and *Rana catesbeiana*, Gastrocnemius‡,§	Water	0.47	0.47	5–15	18
Frog, *Rana pipiens*, Semitendinosus‖	K+	0.56		Minutes	19
	Na+	0.49		Minutes	19
	SO_4^{--}	0.42		Minutes	19
	Sucrose	0.5		Minutes	19
	ATP^{3-}	0.40		Minutes	19
	Ca++	0.02		Minutes	19
Frog, North American Leopard					
Untreated‖	K+	0.12		Minutes	20
Treated with iodoacetate‖	K+	0.7		Minutes	20
Untreated‖	HTO¶	0.6		Minutes	20
Toad, *Bufo marinus*‡,§					
Sartorius	Water	0.5			21
Adductor	Water	0.7			21
Rat heart ventricles, Sprague-Dawley	Water	0.39	0.39–0.33	2–60	13
Rabbit heart, New Zealand white	Water		0.26		13
Rat, skeletal	Water		0.5	~25**	22
Rat, thigh	Water		0.65–0.30	10–35	23
Rat, tibialis anterior	Water		0.44[c], 0.61‖	16	17
Rat tail muscle‡	Water		0.67–0.57	25–500	‡‡
Barnacle, *Balanus nubilus*‖	Water	1.0		Minutes	24
	Urea	1.4		Minutes	24
	Glycerol	1.3		Minutes	24
Cow, clod (shoulder)	Water	0.64	0.64	7–125	25

*This work.
‡Evidence given that muscle samples were kept alive during the experiment.
§Diffusion perpendicular to fiber.
‖Diffusion parallel to fiber.
¶D(HTO) = 2.24×10^{-5} cm^2/s[8].
**Private communication.
‡‡Tanner, J. E., and B. Carew. Unpublished measurements.

of diffusion times, the apparent D are a function of diffusion time. Thus measurements at a single diffusion time must be interpreted with caution.

We see that the present values are in general agreement with D's of water measured for the other muscles, particularly when the measurements are made at a short enough diffusion time so as to obtain a value uninfluenced by the outer membrane, i.e., D_o. D's of other substances show similar reductions relative to their values in ordinary aqueous solution, suggesting that we are observing a simple viscosity effect and not a specific interaction.

Although the D at short time are about a factor of 2 slower than in pure water, they are still much faster than in other types of animal cells, such as red cells (12, 13), liver (14), eggs (15), or eye lens (16). This is probably because most of the dry weight is composed of actomyosin

filaments, which are concentrated in bundles. Calculations by Cleveland et al. (17) show that obstacles of this geometry do not pose a large barrier to diffusion. The surrounding cytoplasm would contain a relatively low solute concentration, and thus diffusion would be fast.

The frog muscle samples studied here were furnished and prepared by Professor Alfred Strickholm of the Department of Physiology, Indiana University. He also provided helpful discussions of the results.

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