WATER IN BARNACLE MUSCLE

III. NMR STUDIES OF FRESH FIBERS AND MEMBRANE-DAMAGED FIBERS EQUILIBRATED WITH SELECTED SOLUTES

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ABSTRACT Water in barnacle muscle has been studied using NMR techniques. Fresh fibers are compared with membrane-damaged fibers treated with solutes that greatly alter fixed charge and total water content. Both water (97%) and solute (3%) protons are visible in continuous wave spectra of oriented fresh fibers. No local field inhomogeneities were detected, nor are cell solutes significantly bound. In pulse experiments, all cell water is visible and exhibits a single exponential decay. In fresh fibers, $T_2 \simeq 40$ ms; faster decaying signals are assigned to immobile and mobile protons on macromolecules. T_1 and $T_{1\rho}$ are frequency dependent. Using equations derived for a two-compartment model with fast exchange, we calculate the following: τ_b, the correlation time for anisotropic rotational motion of bound water; S_b, its order parameter; τ_{ex}, the correlation time for exchange between bound and free fractions; f, the fraction of water bound; and H_r, the grams of water bound per gram of macromolecule. Whereas f varies inversely with total water content, the other parameters are virtually constant, with values: $\tau_b \simeq 1.3 \times 10^{-8}$ s; $\tau_{ex} \simeq 8 \times 10^{-6}$ s; $S_b \simeq 0.06$; and $H_r \simeq 0.1$ g H_2O/g macromolecule. Thus, the NMR relaxation detectable properties of water bound to macromolecules are unaffected by solutes that greatly alter the macromolecular surface charge.

INTRODUCTION

In our studies on muscle cells from the giant barnacle, *Balanus nubilus,* we have become especially interested in the role that various types of intracellular solutes play in regulating cell water content. In the living barnacle fiber, only about half the dry weight is nonsoluble matter, mainly myofilament protein. Of the remaining soluble dry weight, 23% (of the total dry weight) is high molecular weight solids, 23% is small organic solutes, and only 3% is inorganic ions (Clark and Hinke, 1980). In particular, we have investigated the effect of the small organic solutes, which are mainly amino acids and other nitrogenous compounds, on the equilibrium distribution of water and ions in fibers with membranes disrupted by detergent. Briefly, we find that both the net negative fixed charge and the fiber water content increase

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with increasing ionic strength. Although the increased charge is unaltered, the salt-induced swelling is virtually eliminated by addition of certain small organic solutes. In most of our studies we utilized the highly polar nonionic solute, trimethylamine oxide (TMAO), but it was found that amino acids, propionic acid, and glycerol also produce similar results (Clark et al., 1980). Moreover, at identical salt concentrations in the medium, the addition of organic solutes leads to higher concentrations of cations and anions within the fiber, without affecting the net fixed macromolecular surface charge.

These observations have been interpreted in terms of a model where the fixed charge within a fiber is effectively screened in the presence of organic solutes, presumably by permitting a closer association of mobile ions to the charged sites. This raises the question of whether these effects produce any measurable change in the properties of the water hydrating macromolecular surfaces. To answer this question we have employed continuous wave (CW), Fourier transform (FT), and several pulse nuclear magnetic resonance (NMR) techniques to probe the molecular motions of water in fresh fibers and in membrane-damaged fibers equilibrated with various combinations of solutes. The experimental results are presented in two sections: spectral studies on oriented fibers, and pulse experiments; these are followed by our theoretical analysis of the results in which comparisons with alternative theories are made. Finally, we discuss the biological implications of our findings.

In general, our results on fresh fibers agree with several other studies on the molecular motions of cell water. Furthermore, we will show that, regardless of the wide-ranging water content of variously treated membrane-damaged fibers, the amount and behavior of the motionally restricted water (i.e., water restricted in reorientational motion as measured by NMR relaxation) hydrating the cellular macromolecules remains constant. The amount of this rotationally restricted water is less than the amount of translationally restricted water estimated from water self-diffusion measurements on similarly treated fibers, as reported in the fourth paper of this series.[1] A short note on self-diffusion and relaxation studies of fresh barnacle muscle fibers has already appeared (Burnell et al., 1979).

MATERIAL AND METHODS

Fiber Preparation

Muscle fibers used in this study were obtained from the scutal depressor muscles of the giant barnacle, *B. nubilus*. Methods of dissection and of equilibration of membrane-damaged fibers with experimental solutions have been described previously (Clark and Hinke, 1980; Clark et al., 1980). The composition of the solutions employed in these experiments is given in Table I. All solutions were adjusted to pH 7.0.

After equilibration, fibers were blotted free of adhering moisture and were placed, either whole or minced, into NMR tubes; six to fifteen fibers generally contributed to each sample. Wet and dry weights on simultaneously treated companion fibers were always taken. To quantify the fraction of water protons detected by the free induction decay (FID) signal, the water content of these companion fibers was used to estimate the water contained in a known wet weight of fibers in the NMR sample tube. Fibers were maintained at 0°C until measurements were made; all measurements were made at ambient probe temperature (25°C for relaxation studies and 32°C for high resolution studies).

All of the high resolution CW spectra were taken on single fibers confined in glass capillaries to remove contributions to the linewidth from bulk magnetic susceptibility effects arising from asymmetric

[1]Clark, M. E., E. E. Burnell, N. R. Chapman, and J. A. M. Hinke. Manuscript in preparation.

TABLE I
COMPOSITION OF SOLUTIONS USED TO EQUILIBRATE MEMBRANE-DAMAGED FIBERS

Solute	Solution			
	TOO (buffer only)	TMO (salt + buffer)	TO5* (buffer + TMAO)	TM5‡ (salt + TMAO)
Propionic acid	11.5	11.5	11.5	11.5
TRIS	13.5	13.5	13.5	13.5
EGTA	5	5	5	5
Na	0	50	0	50
K	0	150	0	150
Mg	0	10	0	10
Cl	0	220	0	220
TMAO	0	0	500	500

All concentrations in millimolars; all pH = 7.0.
*TO1, TO2 . . . , TO6 have 100, 200 . . . , 600 mM TMAO, respectively.
‡TM1, TM2 . . . , TM6 have 100, 200 . . . , 600 mM TMAO, respectively.

sample shapes. Each fiber was gently drawn by a thread attached to its tendon through a capillary of the same diameter as the fiber. (Contraction of fresh fibers did not occur, since they were dissected in calcium-free Ringer's solution containing 2 mM EGTA.) The capillary was then mounted within an open-ended 5-mm NMR tube by means of washers made from Teflon tape. A region of the sample free of air spaces was selected for study to attain as close ideal cylindrical geometry as possible.

Samples used for studying the effects of macroscopic orientation in the external magnetic field were prepared according to the method described by Fung (1975). Under a low-power microscope, short lengths of fiber were mounted parallel to each other on a Teflon plug designed to fit snugly into a 5-mm thin-walled NMR tube. The plug was held in place with Teflon tape. Orientation of the fibers relative to the magnetic field, H_0, was determined from the position of a pointer, aligned parallel with the long axis of the fibers, that was attached to a glass rod mounted on top of the NMR tube.

NMR Measurements

Most of the CW spectra were obtained at 100 MHz on a Varian XL-100 high resolution NMR spectrometer (Varian Associates, Palo Alto, Calif.), using capillary mounted fibers with the long axis perpendicular to H_0. Spectra were taken either by sweeping the field at a rate of 1 to 4 Hz/s or by accumulating 25 FID scans at a 6-s repetition rate on a Varian 620/L minicomputer and performing a Fourier transform. Samples were usually spun at 50 to 70 Hz.

Spectra of oriented samples mounted on Teflon plugs were taken at 90 MHz on a Bruker SXP 4-100 pulse spectrometer equipped with a B-NC-12 minicomputer (Bruker Spectrospin Ltd., Mississauga, Ont.). In general, 25 FID scans were collected at intervals of at least five times T_1. The FT spectrum was displayed on an oscilloscope on which a cursor permitted measurement of the distance, in Hertz, separating TMAO (methyl) and water proton peaks, and of the shift in peaks observed upon reorientation of the sample.

Pulse measurements at 90 MHz were performed on the Bruker SXP 4-100 spectrometer, while those at Larmor frequencies from 30 to 60 MHz were made on a Bruker (BKR 322-S) pulse spectrometer. The spin-lattice relaxation time, T_1, was measured using a 180°, τ, 90° pulse sequence, sampling the FID with a Bruker pulse-gated integrator; the digitized output was accumulated in a JEOL JRA-5 minicomputer (JEOL [U.S.A.] Inc., Burlingame, Calif.). A linear least squares fit of the regression of ln $(A_\infty - A_\tau)$ vs. τ was calculated by the minicomputer, where A is signal amplitude and τ is the time, in seconds, between pulses. Signals for all other pulse sequences were recorded on a Tektronix storage oscilloscope (Tektronix, Inc., Beaverton, Oreg.) equipped with a Polaroid camera (Polaroid Corp.,

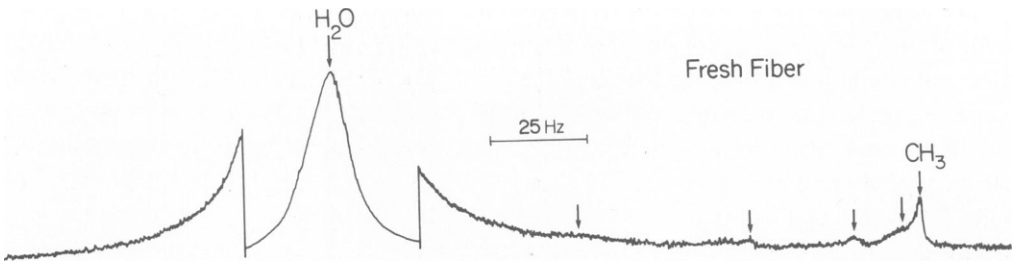

FIGURE 1 High resolution CW ^1H spectrum at 100 MHz of fresh fiber in capillary oriented perpendicular to H_0. The center of the water peak is attenuated 10 times relative to the remaining spectrum. Arrows identify distinct nonaqueous proton peaks. The methyl (CH_3) peak of TMAO and betaine appears at the far right of the spectrum.

Cambridge, Mass.) and were measured manually. The recording apparatus was found to give linear responses to signal intensity within the area of the screen being used.

A conventional two pulse 90°, τ, $180°_{(\pi/2)}$ spin-echo sequence was used for measurements of the spin-spin relaxation time T_2 and, in some experiments, was compared with a 90°, τ, $90°_{(\pi/2)}$ spin-echo sequence (decay time T_3). In both cases, the $\pi/2$ subscript indicates that the phase of the rf carrier of the second pulse is shifted by $\pi/2$ relative to the first pulse.

Measurements of relaxation in the rotating frame ($T_{1\rho}$) were carried out by replacing the BKR 322-S transmitter with a modified Heathkit DX-60 B transmitter (Heath Company, Mississauga, Ont.). Applied angular frequencies, ω_1 ($= \gamma H_1$, where γ is the gyromagnetic ratio and H_1 is the applied field strength at right angles to H_0), ranged from 4.0×10^3 to 2.39×10^5 s^{-1}. In some measurements the rf field was calibrated by measuring the pulse time, τ_p, required to obtain an integral × 180° pulse at the resonance field, H_0, according to the relationship $\omega_1 = n\pi/\tau_p$, where n is an integer; values of ω_1 for at least four values of n were averaged. In other $T_{1\rho}$ experiments, ω_1 was obtained from the relative height of the rf signal from the spin-locking pulse measured with a pick-up coil mounted near the sample. For each point during the decay of the initial FID signal after termination of the H_1 pulse at various times τ, a baseline correction was made with the H_1 pulse off.

RESULTS AND DISCUSSION

A. Studies on Oriented Fibers

1. HIGH RESOLUTION SPECTRA OF CAPILLARY ORIENTED FRESH AND MEMBRANE-DAMAGED FIBERS AND OF EQUILIBRATING SOLUTIONS The CW spectrum of protons in a fresh fiber, obtained at 100 MHz, is shown in Fig. 1. Although it is evident that water contributes the vast majority of visible protons, at least five minor peaks (indicated by arrows) are visible upfield. That with the greatest chemical shift from water (1.5 ppm) is assigned to the methyl protons of trimethylamine oxide (TMAO) and glycine betaine, known to be present in the living fiber (Clark and Hinke, 1980). This identification is based on (*a*) the fact that similar shifts between water and TMAO protons are observed in pure solutions and in fibers equilibrated with media containing TMAO (see below) and (*b*) the reasonably close agreement between the ratio of TMAO + betaine methyl protons to water protons measured under the curves in Fig. 1 with a planimeter (0.005), and that expected from the chemical determination of these solutes in fresh fibers (0.007)(Clark and Hinke, 1980). The remaining minor peaks are probably protons associated with glycine, proline, and other organic solutes present in fresh fibers which, together, have a concentration of ~700 mM (Clark and Hinke, 1980).

The integrated signal from all of these nonwater protons, measured from Fig. 1, represents ~3% of the total proton signal, and may thus contribute a small but measurable amount to the FID amplitude and spin echoes observed during pulse experiments on fresh fibers. These nonwater protons are probably responsible for the 3% of total protons with a relatively long T_2 of 400 ms observed in fresh barnacle muscle by Foster et al. (1976). (We did not observe the slow decay of these protons in our T_2 measurements since our readings were generally taken only until the signal had decayed to between e^{-2} and e^{-3}.) Foster et al. interpret this slowly decaying signal as protons of extracellular water; however, for reasons described below, we do not concur. Their value of T_2 = 400 ms, on the other hand, is consistent with the methyl group linewidth seen in Fig. 1 (~1–2 Hz).

High resolution CW and FT spectra on various solutions and on capillary-mounted, membrane-damaged fibers equilibrated with these solutions were also taken. The data are summarized in Table II. These experiments yielded the following results. Firstly, comparisons

TABLE II

WATER AND TMAO-CH_3 PROTON CHEMICAL SHIFTS IN PARTS PER MILLION MEASURED FROM HIGH RESOLUTION 100 MHz SPECTRA OF SOLUTIONS AND VARIOUSLY TREATED FIBERS

Sample	g H_2O per g nonsolute dry wt‡	Spin rate	σ_{H_2O}*	σ_{CH_3}*	$\sigma_{CH_3}-\sigma_{H_2O}$
		(hertz)			
Fourier transform (FT) spectra (25 scans)					
TOO fiber	11.13	0	0.005		—
TOO fiber	11.13	60	0.005		—
TMO fiber	11.05	0	0.005		—
TO1 fiber	9.93	0	0.000	1.47	1.47
TO2 fiber	10.48	0	0.000	1.53	1.53
TO6 fiber	7.81	0	0.000	1.50	1.50
TO6 fiber	7.81	60	0.010	1.51	1.52
TM6 fiber	7.41	0	0.017	1.52	1.54
TM6 fiber	7.41	(reoriented)	0.012	1.51	1.52
Continuous wave (CW) spectra					
TO6 solution	—	50			1.50
TM6 solution	—	50			1.49
TO1 fiber	9.93	60			1.48
TO3 fiber	8.85	70			1.46
TM3 fiber	8.83	70			1.48
TO6 fiber	7.81	60			1.51
TO6 fiber	7.81	70			1.54
TM6 fiber	7.41	60			1.50
TM6 fiber	7.41	70			1.48
TM6 fiber	7.41	70			1.48
TM6 fiber	7.41	90			1.51
Fresh fiber	5.98	40			1.50

*Chemical shifts are referred to external lock. These are only approximate measurements since no corrections for differences in sample bulk susceptibilities have been made. Such effects will not influence the shifts between water and methyl protons.

‡[g H_2O per g nonsolute dry weight] relates water content to the insoluble solids in the fiber and thus permits comparisons among fibers containing widely different amounts of dissolved solids.

of FT spectra on the same fiber either rotated to different stationary positions relative to H_0 (TM6 fiber) or held stationary and then spun at 60 Hz (T00 and T06 fibers) show identical chemical shifts for the proton peaks. Furthermore, no changes in peak widths occurred. Secondly, when referred to the external lock, the positions of the water peaks from all FT spectra were within 0.017 ppm of each other, regardless of fiber water content or treatment. The positions of the TMAO-methyl peaks on four different fibers were also similar. Thus, the mean shift between TMAO-methyl and water protons on 16 different spectra, from seven different fibers, was 1.50 ± 0.01 ppm (SE). This is the same as the shift of 1.49 to 1.50 ppm observed between methyl and water protons in two TMAO solutions; note that the addition of salt has no effect on this splitting. These results suggest that the relative shielding of water and methyl protons in fibers is not detectably different from that in simple solutions.

All spectra of capillary mounted fibers, both fresh and treated, gave a single, unsplit line for the water of width in rough agreement with the T_2 measurements reported below. Hence, no residual dipolar splitting is detected in the NMR spectra of the water in any of the fibers. Furthermore, the TMAO-methyl peaks were always narrower than those of H_2O, and also gave no evidence of splitting. It is thus unlikely that a significant fraction of these organic molecules is bound to macromolecules in a manner comparable to that of hydration water.

2. MEASUREMENTS OF TEFLON-MOUNTED ORIENTED FIBERS In FT spectra obtained at 90 MHz on fibers with their long axes oriented at angles from 0 to 90° to the direction of the static magnetic field, H_0, the water proton peak underwent large shifts relative to the external lock (Fig. 2). In some instances (TM5, Fig. 2 *b*) splitting of the water signal was also observed. In fibers containing visible amounts of TMAO, the methyl peak also underwent similar shifts (arrows in Fig. 2 *b*). Shifts and linewidths could not be correlated with fiber treatment. To demonstrate the origin of these shifts and splittings, we placed a 2-mm (i.d.) glass capillary filled with water on the Teflon plug. A shift relative to the external lock of over 100 Hz during rotation from 0 to 90° with respect to H_0 was observed, and splittings were seen at both of these orientations. Both shifts and splittings are therefore artifacts arising from bulk susceptibility effects due to sample and Teflon plug geometry. Klein and Phelps (1969) have made similar observations and interpretations about oriented rat phrenic nerve and about moistened twine. It is thus clear that such shifts and splittings as observed here on barnacle muscle fibers, and by Fung (1975) on frog muscle, cannot be used as evidence of anisotropic ordering of water in muscle tissues. In fact, the experiments already reported in section A.1 preclude the existence of such splittings in our experiments.

B. Pulse Experiments

1. COMPONENTS OF DECAY CURVES Before describing and interpreting the results of our pulse experiments, we must analyze the shape of the decay curves obtained, and assign the proton signals observed to various components of the system. The experimental free induction and spin echo decay of a fresh muscle fiber is shown in Fig. 3; this curve agrees closely with that obtained on fresh barnacle muscle by Foster et al. (1976). An initial, very rapidly decaying signal, lasting <100 μs, is assigned to essentially stationary protons on the fiber proteins. This is followed by a "fast-decay" fraction of protons with $T_2 \sim 1.9$ ms. As shown by Foster et al. (1976), these protons do not exchange with D_2O. We concur in their interpretation that this signal represents mobile protons on tissue proteins and lipids. As

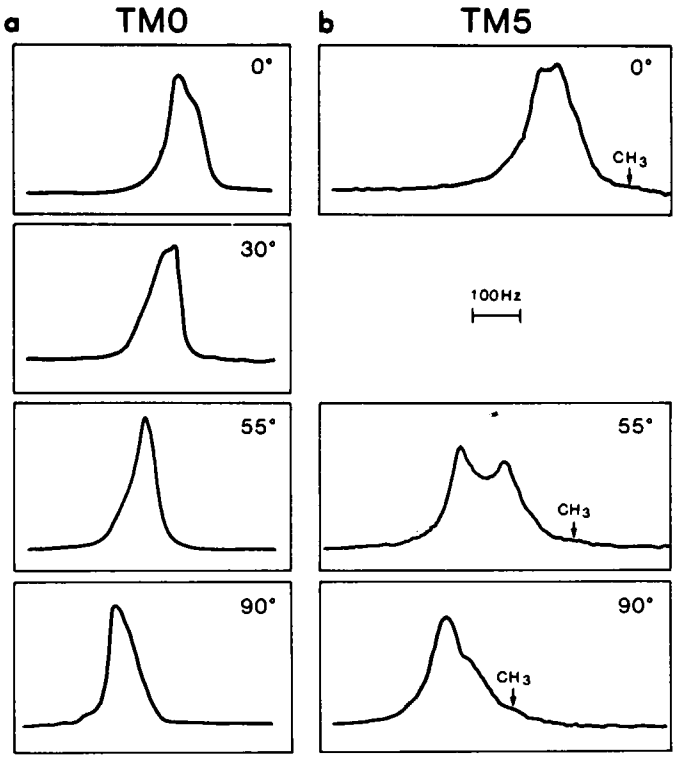

FIGURE 2 FT ^{1}H spectra at 90 MHz. of single fibers oriented with their long axes at varying angles to H_0. All frequency scales for each fiber are coincident. (*a*) TMO – fiber treated with salt only; (*b*) TM5 – fiber treated with salt plus 0.5 M TMAO. Arrows labeled CH_3 indicate position of TMAO methyl proton signal.

Bloom et al. (1977) showed in the case of model-membrane systems, relatively long-lasting free induction signals can be expected from very mobile protons, such as those in membrane lipids or in methyl groups projecting from the surfaces of stationary molecules. Both of these proton components were also observed in membrane-damaged fibers. In all of our pulse experiments, decay curves at times >10 ms yielded a single exponential main decay fraction, which we have taken as the total water proton decay signal. The relative sizes and the T_2 values for the "fast-decay" fraction and the "main-decay" fraction of protons in our variously treated fibers are given in Table III, together with data from several other pulsed NMR studies of muscle tissue.

As shown in Table III, we observe no "slow-decay" fraction equivalent to that found in vertebrate muscle, even when the signal is allowed to decay to ~3% of its initial amplitude (see the "before" curve in Fig. 4). This fraction of protons has been ascribed by others (Belton et al., 1972; Belton and Packer, 1974; Hazlewood et al., 1974) to extracellular water that has relatively little motional restriction. The extracellular space of single fibers of barnacle muscle contains ~6% of the fiber water, located, however, in deep clefts filled with a collagen-like material (Brigden et al., 1971). We assume that this water, owing to the presence of insoluble protein, relaxes similarly to the intracellular water and is not readily distinguishable from it.

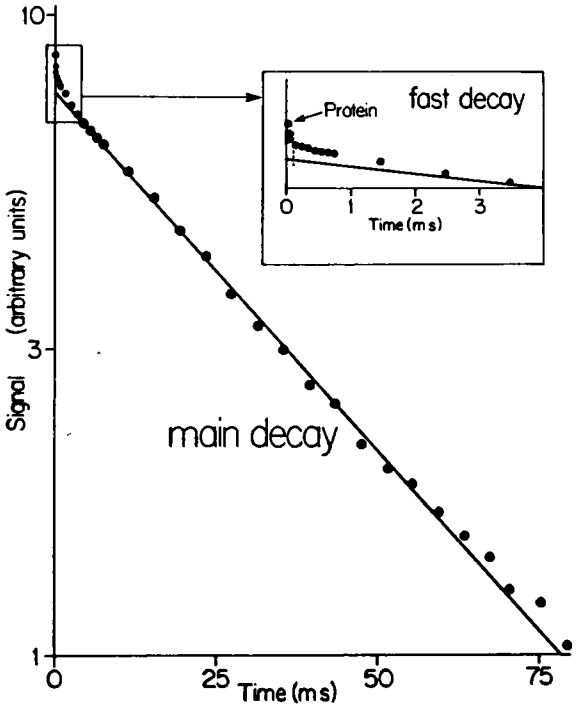

FIGURE 3 Experimental free induction decay. The signal up to 1 ms is the free induction decay following a $\pi/2$ rf pulse. That after 1 ms is obtained using the 90°, τ, $180°_{(\pi/2)}$ spin-echo sequence. Our interpretation of the decay fractions is discussed in the text, and parameters for the fast and main decay fractions are in Table III.

TABLE III

T_2 RELAXATION TIMES AND RELATIVE SIZES OF PROTON COMPARTMENTS IN VARIOUS MUSCLE TISSUES AT ~25°C

Water fraction	Slow		Intermediate		Fast		Reference
Tissue	T_2		T_2		T_2		
	(milli-seconds)	*(percent)*	*(milli-seconds)*	*(percent)*	*(milli-seconds)*	*(percent)*	
Frog gastrocnemius	168	15	38	67	10	18	Belton et al., 1972
Frog gastrocnemius	147	17	44	70	7	12	Belton and Packer, 1974
Rat gastrocnemius (90°, τ, 180°)	155 ±13	9.8 ±1.4	43.7 ±1.0	83.0 ±1.6	0.42 (<5 ms)	7.2 ±1.1	Hazlewood et al., 1974
Rat gastrocnemius (CPMG)	196 ±34	10.0 ±1.3	44.7 ±0.8	82.0 ±1.1	—	8.1 ±0.4	Hazlewood et al., 1974
Barnacle-fresh (CPMG)	400	3	35	91–94	0.3–4	3–6	Foster et al., 1976
Barnacle: fresh	—	—	38.8	94.4	1.9	5.6	This study
Barnacle: TOO (buffer only)	—	—	63.0	94.7	1.0	5.3	This study
Barnacle: TMO (salt + buffer)	—	—	126.6	96.4	0.9	3.6	This study

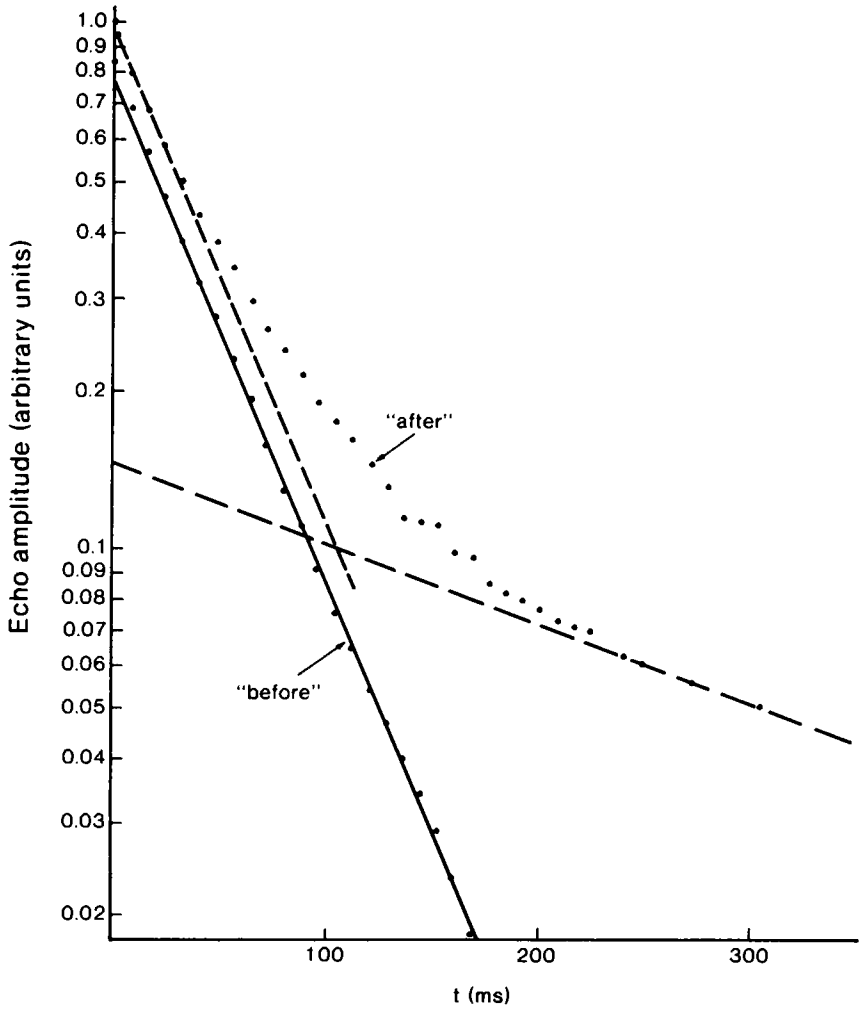

FIGURE 4 Spin-echo decay of a single sample of blotted whole fresh fibers before and after addition of a few drops of barnacle Ringer's solution, measured by a sequence of 90°, τ, $180°_{(\pi/2)}$ pulses, at repetition rates of 4 and 10 s, respectively. T_2 for the "before" measurement is 0.0455 ± 0.0004 s. Note that in the "after" measurement the intercept of the slow component at $t=0$ about equals the difference between the two initial FID amplitudes, and the slope gives a T_2 of 0.286 ± 0.010 s.

To test this hypothesis, we measured the T_2 decay of a single, well-blotted intact fresh muscle fiber before and after addition of barnacle Ringer's solution to the NMR tube. As shown in the "after" curve of Fig. 4, when salt solution fills the crevices in the folded fiber the T_2 decay becomes nonexponential and the amplitude of the new "slow-decay" signal, obtained from extrapolation to zero time, corresponds with the increase in initial signal amplitude from the added Ringer's solution. It thus seems reasonable that the "slow-decay" fraction in vertebrate muscle, which has no deep, protein-filled clefts and has 10–20% of its water located extracellularly, does indeed arise from such water.

To further demonstrate that the main (intermediate) decay fraction actually represents all

of the water in barnacle muscle, we compared the known water content of various fibers (obtained from the measured hydration of companion fibers) with the height of the FID signal of the main decay fraction. Within experimental error, all of the protons of the fiber water plus those known to be present on organic solutes were included in the main decay fraction. Furthermore, errors in estimating the slopes in our relaxation experiments, that were introduced by ignoring the contribution from the slowly decaying signal of organic solute protons in fresh fibers and TMAO-treated fibers, are less than the errors of the measurements themselves.

2. EFFECT OF PULSE SEQUENCE ON OBSERVED TRANSVERSE RELAXATION The decay times of the main decay fraction of variously treated fibers obtained from 90°, τ, $180°_{(\pi/2)}(T_2)$ and from 90°, τ, $90°_{(\pi/2)}(T_3)$ experiments are compared in Table IV. There is no difference between the values of T_2 and T_3 (obtained from two pulse experiments) for any of the fibers. If nonzero time-averaged dipolar splittings were present, the echo decay of T_3 would not be affected, while that of T_2 would be nonexponential, and the time constant, T_2^*, for echoes to decay to $1/e$ of their original magnitude, would be less than T_3 (Woessner and Snowden, 1969). That T_2 and T_3 are the same provides further evidence that there is no residual dipolar splitting in these fibers.

3. EFFECT OF FIBER TREATMENT ON T_1, T_2 and $T_{1\rho}$ The values obtained for T_1 (180°, τ, 90°) and T_2 (90°, τ, $180°_{(\pi/2)}$) for the main decay fraction on variously treated fibers at several frequencies are shown in Table V. Each experiment indicated in the first column identifies a completely separate study, using a different barnacle and freshly made solutions. As expected from earlier studies (see Table 3, p. 104, in review by Cooke and Kuntz, 1974), both T_1 and T_2 are reduced compared with simple aqueous solutions, and T_2 is an order of magnitude less than T_1. For any given treatment, T_1 is frequency dependent, being longer at higher frequencies, whereas T_2 is frequency independent (note especially Experiment XV). This frequency independence of T_2 provides evidence that there is no inhomogeneity in the magnetic susceptibility within the fiber.

Studies of relaxation in the rotating frame, $T_{1\rho}$, have been performed at ν_0 = 30 MHz on

TABLE IV

TIME FOR DECAY OF MAGNETIZATION TO $1/e$ AFTER 90°, τ, $180°_{(\pi/2)}(T_2)$ AND 90°, τ, $90°_{(\pi/2)}(T_3)$ PULSE SEQUENCES IN MINCED BARNACLE MUSCLE FIBERS AFTER VARIOUS TREATMENTS

	Fiber treatment				
	Fresh	TM6	TMO	TO6	TOO
g H_2O per g nonsolute dry weight (n = 10)	5.77 (est.)	9.00 ± 0.28	14.23 ± 0.90	7.83 ± 0.29	6.35 ± 0.22
T_2 (90°, τ, $180°_{(\pi/2)}$)*	43 ms ± 1	70 ms ± 1	128 ms ± 2	67 ms ± 1	55 ms ± 1
T_3 (90°, τ, $90°_{(\pi/2)}$)*	43 ms ± 1	74 ms ± 1	129 ms ± 1	70 ms ± 1	55 ms ± 1

(Standard errors of individual slopes are given: $r^2 \geq 0.995$.)

*4-ms increments in τ spacings; slope calculated from echoes at $t \geq 20$ ms.

TABLE V
EFFECT OF FIBER TREATMENT AND LARMOR FREQUENCY ON T_1 AND T_2

Experiment	Preparation	g H_2O per g total solid*	g H_2O per g nonsolute dry weight	ν_0	T_1 (180°, τ, 90°)	T_2 (90°, τ, 180°$_{\pi/2}$)
				(megahertz)	*(seconds)*	*(seconds)*
XI	Fresh	3.00 (est)	5.77	30	0.72 ± <0.01	0.043 ± 0.001
XII	Fresh	2.97 ± 0.01	5.71 ± 0.02	30	0.66 ± <0.01	0.040 ± 0.001
XV	Fresh	3.04 ± 0.05	6.14 ± 0.20	30	—	0.039 ± <0.001
XV	Fresh	3.04 ± 0.05	6.14 ± 0.20	60	—	0.041 ± 0.001
I	Fresh	3.45 ± 0.05	6.63 ± 0.10	90	1.24 ± 0.03‡	—
II	Fresh	3.32 ± 0.14	6.38 ± 0.27	90	1.30 ± 0.08‡	—
XI	TM6	4.87 ± 0.08	9.00 ± 0.28	30	0.82 ± <0.01	0.070 ± 0.001
XII	TM6	3.95 ± 0.04	6.29 ± 0.10	30	0.72 ± 0.01	0.074 ± 0.002‡
XV	TM5	4.27 ± 0.15	6.26 ± 0.33	30	—	0.076 ± 0.003
XV	TM5	4.27 ± 0.15	6.26 ± 0.33	60	—	0.076 ± 0.001
I	TM5	2.94 ± 0.06	3.88 ± 0.10	90	1.11 ± 0.01‡	—
VI	TM5	4.18 ± 0.02	6.37 ± 0.05	90	1.08 ± 0.02	—
XI	TO6	4.84 ± 0.11	7.83 ± 0.29	30	—	0.067 ± 0.001
XII	TO6	5.09 ± 0.09	8.50 ± 0.25	30	0.82 ± 0.01	0.085 ± 0.001
XV	TO5	5.12 ± 0.08	7.36 ± 0.16	30	—	0.083 ± 0.001
XV	TO5	5.12 ± 0.08	7.36 ± 0.16	60	—	0.083 ± 0.003
XI	TOO	6.00 ± 0.19	6.35 ± 0.22	30	—	0.055 ± <0.001
XII	TOO	9.29 ± 0.22	10.16 ± 0.27	30	0.93 ± 0.01	0.094 ± 0.001
XV	TOO	6.01 ± 0.05	6.15 ± 0.05	30	—	0.072 ± <0.001
XV	TOO	6.01 ± 0.05	6.15 ± 0.05	60	—	0.073 ± 0.004
II	TOO	6.27 ± 0.28	6.66 ± 0.31	90	1.59 ± 0.04	—
XI	TMO	10.68 ± 0.50	14.23 ± 0.90	30	1.25 ± 0.01‡	0.128 ± 0.002
XII	TMO	11.20 ± 0.79	15.17 ± 1.48	30	1.28 ± 0.01	0.134 ± 0.015‡
XV	TMO	8.37 ± 0.19	9.94 ± 0.27	30	—	0.116 ± 0.001
XV	TMO	8.37 ± 0.19	9.94 ± 0.27	60	—	0.111 ± 0.004
I	TMO	8.59 ± 0.37	10.75 ± 0.58	90	1.71 ± 0.01‡	—
II	TMO	7.10 ± 0.19	8.51 ± 0.28	90	1.77 ± 0.03	—

*Mean ± standard error of $n \geq 5$ measurements on companion fibers.
‡Mean ± standard error of two replicate decay curves on same sample. Other errors are for slope of a single decay curve.

variously treated fibers as a function of H_1 field strength. The $T_{1\rho}$ results for fresh fibers are shown, together with those for T_1 and T_2, in Fig. 5. Similar sets of values were obtained for TM6, TM3, TM0, T06, and T00 fibers (Table VI). In all cases, a large change in $T_{1\rho}$ over the frequency range studied was observed. Comparisons between treatments indicate that, in general, T_1, T_2, and $T_{1\rho}$ increase with fiber water content. This is shown graphically for values of T_2 in Fig. 6, where the regression for water content relative to nonsolute (primarily myofilament) dry weight is shown.

C. Discussion of Water Motions in Barnacle Muscle

Numerous NMR investigations of the molecular motions of water in biological systems have been conducted in recent years. Much of the work before 1974 has been extensively reviewed (Cooke and Kuntz, 1974; Kuntz and Kauzman, 1974). There is general agreement that the

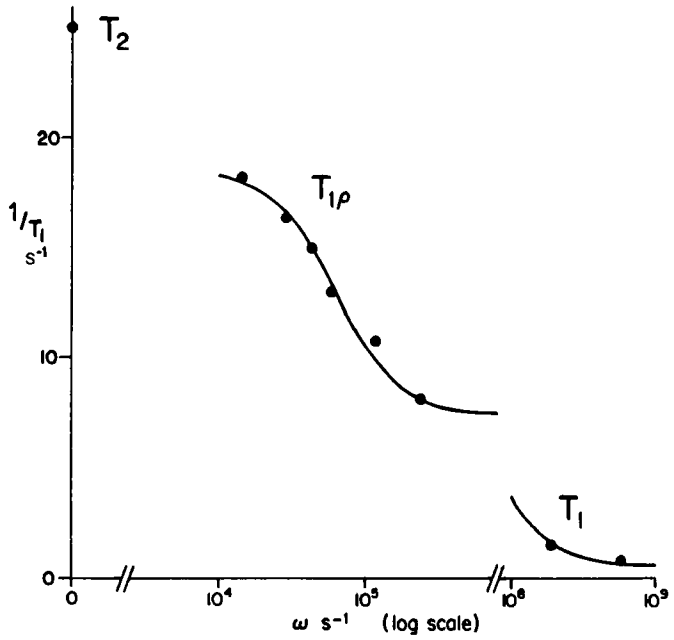

FIGURE 5 Relaxation results for fresh fibers at various frequencies, ω, where $\omega = \omega_0$ for T_1 measurements and $\omega = \omega_1$ for $T_{1\rho}$ and T_2 measurements. For $T_{1\rho}$ and T_2 experiments, $\omega_0/2\pi = 30$ MHz. The solid lines were drawn using parameters calculated from Eq. A9 and reported in Table VII.

TABLE VI
FREQUENCY DEPENDENCE OF $T_{1\rho}$ FOR VARIOUSLY TREATED FIBERS

Fiber	Fresh fiber		TM6		TM3		TMO		TO6		TOO	
g H_2O per g nonsolute dry wt	3.95*		6.29		8.06		15.17		8.50		10.16	
	ω_1	$T_{1\rho}$	ω_1	$T_{1\rho}$	ω_1	$T_{1\rho}$	ω_1	$T_{1\rho}$	ω_1	$T_{1\rho}$	ω_1	$T_{1\rho}$
	1.43	0.055	0.77	0.103	0.73	0.144	0.40	0.225	0.73	0.113	0.73	0.126
	2.94	0.061	1.43	0.109	1.45	0.158	0.79	0.240	5.92	0.158	5.86	0.178
	4.34	0.067	2.94	0.124	2.94	0.172	1.78	0.252	23.9	0.209	19.9	0.249
	5.86	0.077	4.34	0.129	5.86	0.190	2.42	0.265				
	11.69	0.093	5.86	0.138	5.92	0.196	3.53	0.284				
	23.9	0.123	8.66	0.150	12.04	0.221	4.69	0.301				
			11.69	0.157	23.9	0.248	7.03	0.323				
			23.4	0.184			9.71	0.345				
							15.19	0.383				
							21.6	0.405				

ω_1 values are in units of $10^4 s^{-1}$ and $T_{1\rho}$ values are in seconds.
*For fresh fibers, in addition to the nonsolute dry weight, the soluble macromolecules in the sarcoplasm have been included in calculating the relative water content of the fiber (see Clark and Hinke, 1980).

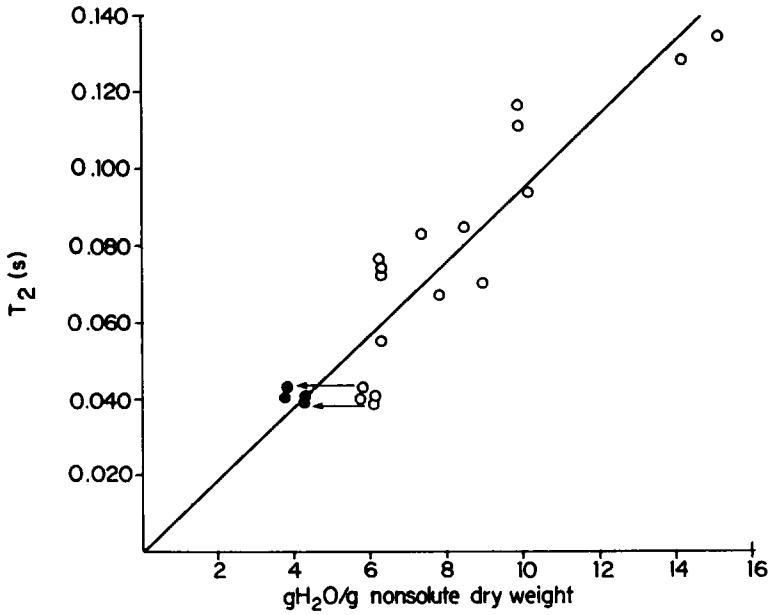

FIGURE 6 Linear relationship of T_2 vs. fiber water content expressed in terms of gram water per gram nonsolute dry weight. (The regression coefficient, r^2, is 0.79). Note that the four fibers with lowest T_2 are fresh fibers that also contain soluble macromolecules; correction of hydration for these would place these four points much nearer the regression line, as indicated by arrows.

following observations are qualitatively characteristic of those living tissues so far investigated.

(*a*) Unlike pure liquids, where the spin-lattice (T_1) and spin-spin (T_2) relaxation times are similar, in systems containing macromolecules, T_2 is much less than T_1. In living systems, T_1^{-1} is about five times larger than in bulk water and T_2^{-1} is about fifty times larger. (*b*) T_1 and $T_{1\rho}$ are frequency dependent. (*c*) The mean self-diffusion coefficient of water in cells containing 75–80% water is 50–60% of that in bulk water (e.g., Abetsedarskaya et al., 1968; Finch et al., 1971; Chang et al., 1972, 1973; Rorschach et al., 1973; Cleveland et al., 1976). Measurement and interpretation of the self-diffusion coefficient of water in barnacle muscle is treated in detail elsewhere (Burnell et al., 1979; Clark et al.[1]). (*d*) In some preparations, T_1, $T_{1\rho}$, and T_2 decays are nonexponential (e.g., Belton et al., 1972; Hazlewood et al., 1974; Knispel et al., 1974; Shporer et al., 1976). These four observations have led to several interpretations of the behavior of water in biological systems.

The nonexponential decay of $T_{1\rho}$ and especially of T_2 observed in some preparations is generally ascribed to water compartments within the tissue, among which molecular diffusion is slow relative to T_i, where i is the decay process being measured. In other words, the decay is interpreted as superimposed signals from two or more independently relaxing populations of water molecules (Belton et al., 1972; Diegel and Pintar, 1975; Shporer et al., 1976). In barnacle muscle, however, for the reasons discussed in section B.1 above, we consider that the main-decay fraction of protons, which gives a single exponential decay, comprises all of the water in the fiber.

The frequency dependence of T_1 and $T_{1\rho}$ observed here in barnacle muscle is similar to that found previously in other tissues (Held et al., 1973; Outhred and George, 1973; Knispel et al., 1974; Fung and McGaughy, 1974; Block and Maxwell, 1974; Finch and Homer, 1974; Civan and Shporer, 1975; Fung et al., 1975; Zipp et al., 1976). It leads to the inequality between T_1 and T_2, and can be due to several relaxation mechanisms. Those that are likely to be important in biological systems are: the presence of paramagnetic species; the diffusion of water molecules through local magnetic field gradients; and dipolar coupling.

There seems to be consensus that paramagnetic ions do not contribute significantly to the reduced T_2 of water protons in living tissues (Finch et al., 1971; Hazelwood, 1979). The effects of dissolved oxygen are small and relatively constant from one preparation to the next. Relaxation via diffusion of water molecules through local magnetic field gradients (that arise from inhomogeneities in magnetic susceptibility in the tissue) would be proportional to the strength of the external magnetic field; hence the spin-spin relaxation rate, T_2^{-1}, should vary with the square of the resonance frequency (Abragam, 1961). We observe no frequency dependence of T_2 in barnacle muscle, however (Table V), and thus conclude that dipolar coupling is the dominant relaxation mechanism of water protons in this system.

1. CHOICE OF A MODEL Two major models for water proton relaxation via dipolar coupling have been proposed in biological systems, both of which envision two (or more) aqueous fractions that exchange rapidly on the time scale of an NMR relaxation measurement. In one model (Resing et al., 1976), a very small proportion (~0.1%) of water is irrotationally bound to solid surfaces, and relaxation is governed by the strong collision limit (Ailion, 1971). The other model, which has been adopted by numerous investigators, proposes a somewhat larger fraction of motionally restricted water with less definitive boundaries, the relaxation of which is governed by the weak collision limit (Ailion, 1971).

Our model is based on that of Diegel and Pintar (1975), and falls within the second category, but contains some important differences. It assumes a two-component system consisting of a fraction of water with restricted anisotropic motion owing to its association with the assumed stationary surfaces of macromolecules (the bound water[2]) and the remaining free water which is assumed to undergo isotropic motions similar to those of water molecules in dilute solutions; molecular exchange between the two components is sufficiently rapid to give a single relaxation time for all relaxation processes observed (Zimmerman and Brittin, 1957). Our model differs from other two-compartment models based on weak collision theory in being more general; it assumes only that the free water tumbles isotropically, whereas the bound water may exhibit anisotropic motion, described by an order parameter, S_b. If motion in the bound fraction is isotropic, the order parameter goes to zero, and we obtain the model for the fast exchange limit described by Zimmerman and Brittin (1957).

The theoretical argument for the derivation of our model is given in the Appendices. The

[2]The term "bound water" has been given many meanings. Throughout this paper, it signifies that water which has measurably restricted rotational motions owing to its association with slowly moving or stationary molecules, mainly proteins. It does not imply any particular thermodynamic state. Similarly, as shown later in this paper, the nonbound or "free water" described in this simple model is not necessarily thermodynamically uniform; indeed, in some instances it is distinctly unlikely to be so. "Free water," then, must be taken only as water without demonstrable rotational restrictions.

model predicts four independent relaxation processes, each characterized by an independent correlation time. The predicted relaxation behavior (Eqs. A9 and A17) is given by

$$T_i^{-1} = (1 - f)T_i(a)^{-1} + f(1 - S_b^2)T_i(b)^{-1} + f(1 - f)S_b^2T_i(ex)^{-1} + f^2S_b^2T_i(lf)^{-1}, \quad (1)$$

where f is the fraction of water bound and i ($\equiv 1$, 2, or 1ρ) labels the relaxation time as T_1, T_2, or $T_{1\rho}$. The terms $T_i(j)$ are given by Eqs. A10–A12, which describe the relaxation for each process (labeled by j and described below) in terms of its correlation time τ_j. The first (a) term in Eq. 1 describes the relaxation of the fraction $(1 - f)$ of free water which is assumed to be tumbling isotropically with correlation time τ_a. The second (b) term describes the relaxation of the anisotropically tumbling bound fraction with correlation time τ_b. Provided that the correlation time for exchange between free and bound fractions, τ_{ex}, is much less than τ_a and τ_b, such exchange serves to modulate the dipolar Hamiltonian between zero (the average over the motions of the free water) and the average over the anisotropic bound motion, $\langle \mathcal{H} \rangle_b$, and hence gives a contribution to relaxation, the third (ex) term in Eq. 1. Even after averaging over these three motions, there remains a residual dipolar Hamiltonian ($= f\langle \mathcal{H} \rangle_b$) which can be modulated by an additional low frequency motion (assumed isotropic) with correlation time τ_{lf}, yielding the fourth (lf) term in Eq. 1. Exchange of protons between different water molecules qualifies as a candidate for this low frequency motion.

Each term in Eq. 1 will contribute a dispersion to a plot of T_i^{-1} vs. frequency (e.g., dispersion of $T_{1\rho}$ values in Fig. 5). If all four dispersions could be measured, their heights would give the terms $(1 - f)\tau_a$, $f(1 - S_b^2)\tau_b$, $f(1 - f)S_b^2\tau_{ex}$ and $f^2S_b^2\ \tau_{lf}$, while their characteristic cut-off frequencies would give the correlation times τ_a, τ_b, τ_{ex}, and τ_{lf}. In our analysis we shall assume values for τ_a and τ_{lf} and obtain values of f, S_b, τ_b, and τ_{ex}.

2. PARAMETERS OBTAINED FROM APPLICATION OF THIS MODEL In applying this model to our relaxation data we have made the following assumptions and assignments. We assume, as have Diegel and Pintar (1975), that τ_{ex} is much larger than τ_a or τ_b. We thus take the frequency dispersion of relaxation determined by T_1 measurements as due to motions of the bound water (τ_b) and that determined by $T_{1\rho}$ measurements as due to exchange between the two fractions (τ_{ex}). As stated above, motions in the free water fraction are assumed to be isotropic and values of T_1 in this fraction are taken to be the same as the values of T_1 measured on the appropriate equilibrating solutions, all of which were very close to the value for pure water. Since the correlation time in bulk liquid water, τ_a, is $\sim 3 \times 10^{-12}$ s (Eisenberg and Kauzman, 1969), the frequency of molecular motions in the free fraction is much greater than the angular frequencies of either the external or applied fields (i.e., $\omega_1\tau_a \ll \omega_0\tau_a \ll 1$); hence, within the frequency range studied, Eqs. A9–A12 indicate no frequency dependence of the relaxation rates in this fraction.

The respective constants [$Kf(1 - S_b^2)$ and $Kf(1 - f)S_b^2$] obtained by fitting our T_1 and $T_{1\rho}$ experimental results to Eqs. A9–A12 are shown in Table VII, together with the values of τ_b, τ_{ex}, f, and S_b derived from them. The theoretical fit of the T_1 and $T_{1\rho}$ relaxation dispersions for fresh fibers is shown by the solid lines in Fig. 5. We note that the order parameter, S_b, although small, is not zero in the three treatments for which it is calculated. Additionally, assuming that only surface water is partially oriented, the values of S_b obtained are consistent with the deuterium NMR splittings observed for D_2O in various model bilayer systems (e.g. Finer [1973]; Abdolall et al. [1977]; Niederberger and Tricot [1977]).

TABLE VII
PARAMETERS OBTAINED FROM ANALYSIS OF THE RELAXATION RESULTS

	Fiber treatment					
	Fresh Fiber	TM6	TM3	TMO	TO6	TOO
g total H_2O per g nonsolute dry wt	3.95*	6.29	8.06	15.17	8.50	10.16
$Kf(1 - S_b^2)$ (s^{-2})	$2.79 \pm 0.17 \times 10^8$	$2.16 \pm 0.13 \times 10^8$		$0.76 \pm 0.09 \times 10^8$		
τ_b (seconds)	$1.31 \pm 0.12 \times 10^{-8}$	$1.06 \pm 0.11 \times 10^{-8}$		$1.34 \pm 0.25 \times 10^{-8}$		
$Kf(1 - f)S_b^2$ (s^{-2})	$9.3 \pm 0.7 \times 10^5$	$3.5 \pm 0.4 \times 10^5$	$1.8 \pm 0.3 \times 10^5$	$1.32 \pm 0.15 \times 10^5$	2.8×10^5	3.0×10^5
τ_{ex} (seconds)	$8.0 \pm 0.8 \times 10^{-6}$	$8.1 \pm 1.1 \times 10^{-6}$	$9.3 \pm 1.7 \times 10^{-6}$	$9.4 \pm 1.0 \times 10^{-6}$	10.4×10^{-6}	9.7×10^{-6}
f	0.018	0.014		0.005		
S_b (from T_1 and $T_{1\rho}$)	0.06	0.04		0.04		
S_b (from T_2)	0.02	0.02		0.06		
H_r‡	0.07*	0.09		0.07		

*For fresh fibers, in addition to the nonsolute dry weight, the soluble macromolecules in the sarcoplasm have been included in calculating the relative water content of the fiber (Clark and Hinke, 1980).
‡$H_r = f$. g total water/g macromolecule = g bound water/g macromolecule.

In all treatments it was found that the curve fitted to the $T_{1\rho}$ experimental results does not extrapolate to the experimental value of T_2 when $\omega_1 = 0$. This is to be expected if anisotropic motion exists in the bound water fraction, as discussed above and in Appendix A. The exponential free induction and spin echo decays, the equality of T_2 and T_3, and the absence of splitting in the frequency spectrum all suggest the presence of an additional low frequency relaxation mechanism which reduces $\langle \mathcal{H} \rangle_{av}$ to zero with a correlation time, τ_{lf}, greater than τ_a, τ_b, or τ_{ex}. This mechanism is probably either the exchange of protons between water molecules, or the diffusion of water between domains with different (random) orientations relative to H_0. With respect to the first possibility, at pH 7.0 and 25°C, the correlation time for proton exchange, τ_{pe}, is 1.8×10^{-3} s (Meiboom, 1961). The second possibility, which has been suggested for several systems by Woessner and his co-workers (Woessner et al., 1969, 1970; Woessner and Snowden, 1973; Hazlewood et al., 1974) will only be observable with proton magnetic resonance if the time constant for such diffusion, τ_d, is less than τ_{pe}. Since deuterium relaxation is unaffected by proton exchange, experiments on fibers partially substituted with D_2O would provide definitive information about the low frequency motions.

As described in Appendix A, if τ_{lf} is known it is also possible to calculate the order parameter, S_b, by the difference, $1/T_2'$, between the value of $1/T_2$ obtained by extrapolation of the $T_{1\rho}$ relaxation dispersion data to $\omega_1 = 0$ (Eqs. A9–A12) and the actually observed value of $1/T_2$. As a first approximation, we take $\tau_{lf} \equiv \tau_{pe} = 1.8 \times 10^{-3}$ s, and obtain the second set of values for S_b shown in Table VII. They are of a similar order to those obtained from the T_1 and $T_{1\rho}$ results. Considering the approximations involved, we take this general agreement as support for the usefulness of our model.

Finally, in Table VII, from f, the bound water fraction, we calculate a value, H_r, the water hydrated, or "bound," to the macromolecular surface. Since f is inversely correlated with water content, this hydration water is constant and does not depend on fiber treatment.

Additionally, the order parameter S_b is small and roughly constant for all treatments. When S_b is constant and f is small (such that $[1 - f] \sim 1$) and is inversely related to water content, and when the $T_i(a)^{-1}$ and $T_i(lf)^{-1}$ terms in Eq. 1 are not major contributors to T_i^{-1}, the model (Eq. 1) predicts a linear dependence of the relaxation times on the total water content in a fiber (g H_2O/g macromolecular dry weight); this has indeed been found experimentally and is shown for T_2 in Fig. 6.

3. ALTERNATIVE MODELS There is no consensus in the literature about the molecular motions contributing to the proton relaxation characteristically observed for water in biological systems. Our two-compartment model, in which each water fraction has a mean correlation time and exchanges with the other fraction, adequately fits the T_1 and $T_{1\rho}$ dispersions given in Fig. 5 and Table VI, and each of the correlation times used in our interpretation has been assigned a physical meaning. Several authors have argued for the presence of a complex distribution of exponential correlation functions, such as a single, continuous log-Gaussian distribution (Held et al., 1973, working with frog muscle) or two or more log-Gaussian distributions (Zipp et al., 1976, working with frozen erythrocytes) or a bimodal log power distribution (Outhred and George, 1973, working on toad muscle). In the absence of physical justification for such distributions, however, our simpler model seems more appropriate. This is not to say that our results could not be better fit by assuming an expanded distribution of correlation functions or by a nonexponential correlation function.

We would point out, however, that the alternative model for water relaxation in barnacle muscle proposed by Resing et al. (1976) cannot be entirely ruled out by our results. In this model, where a small fraction (~0.1%) of the water is irrotationally bound to the macromolecular surface, $\langle \mathcal{H} \rangle_b$ becomes equal to $\mathcal{H}$ (0); Eq. A13 no longer holds, and it is necessary to apply the theory appropriate for the strong collision limit for $T_{1\rho}$ (Ailion, 1971). This model has been discounted by Koenig and co-workers (1975), however, as not explaining water relaxation in protein solutions where the relaxation mechanism is assumed to be the isotropic tumbling of the protein molecules. Indeed, as Walmsley and Shporer (1978) have recently demonstrated, in such systems the results are explainable if the water is not irrotationally bound. In the case of muscle fibers, and especially those with damaged membranes from which the soluble proteins have been removed and hence where there is little or no macromolecular motion, the Resing model does not readily explain the frequency dependence of T_1. It is possible but unlikely that local motions of the surfaces of myofilaments and other cell solids could provide the necessary relaxation mechanism. The main argument against the irrotationally bound water model is that it is very improbable that only 0.1% of the water molecules are bound and that, when bound, they have no motion for times of the order of 10^{-5} s. Even in fully H-bonded ice, the mean rotational correlation time is $\sim 10^{-5}$ s (Eisenberg and Kauzman, 1969).

In our analysis we have neglected effects of cross-relaxation between bound water and protein protons (Edzes and Samulski, 1978). Such effects might be expected to modify the relaxation parameters for the bound water fraction.

D. Biological Implications

These studies suggest that the fraction of motionally restricted (hydration) water in fresh barnacle muscle fiber is ~2% of the fiber water. This is well within the upper limit of 5% set

by the experimental error in the Raman spectroscopy studies of Pezolet et al. (1978); these workers failed to observe any significant difference in the O—H (or O—^{2}H) stretching modes between salt solutions and water in unfrozen barnacle fibers. As pointed out by these workers (and others cited by them), however, as much as 20–30% of muscle water may be so affected by macromolecular surfaces that it does not freeze readily and has reduced solvent capacity.

Like tissues of other marine invertebrates, barnacle muscle fibers contain large quantities (500–700 mM) of nitrogenous organic solutes, the functions of which are largely unknown (Clark and Hinke, 1980, and references therein). This is the first study in which NMR spectra of the protons of these solutes have been reported (Fig. 1). We are reasonably confident that the proton peak shifted 1.5 ppm to high field from that of water protons arises from the methyl groups of TMAO and betaine, present at 82 mM in these fibers (Clark and Hinke, 1980). The relatively narrow linewidth of this signal suggests that these solutes are not significantly bound to solid surfaces in the cell, and that their remarkable effect in regulating excessive swelling in the presence of salts (Clark et al., 1980) may not be mediated via direct association with myofilament proteins or other cell solids.

The results summarized in Table VII clearly indicate that the fraction (f) of motionally restricted (bound) water decreases when fibers swell after treatment with salt, thus yielding a constant macromolecular hydration (H_r). Furthermore, the constancy (within error of the measurements) of τ_b, τ_{ex}, and S_b for the various fibers suggests that the properties of the motionally restricted water (as measured by NMR relaxation) do not vary with fiber treatment: neither with increasing ionic strength, which greatly increases net fixed charge on the fiber macromolecules, and hence causes fiber swelling; nor by the addition of organic solutes such as TMAO, which prevent salt-induced swelling. Whatever effects these solutes may have at the macromolecular surface, they are apparently not reflected in changes as measured by NMR relaxation in the properties of the bound water. It is possible, of course, that they affect the properties of the nonfreezing water that comprises 20% or more of fresh fiber water (Pezolet et al., 1978). In this regard we should note that the self-diffusion coefficients measured via field gradient NMR methods (Burnell et al., 1979; footnote 1) indicate a larger amount of motionally restricted water than do the present relaxation results. These observations indicate that a measure of the amount of restricted water has a meaning which depends on the experiment performed; for relaxation, it is mainly the amount of water that is not tumbling isotropically; for diffusion, it represents water that is restricted in its translational motion. Both relaxation and diffusion values will be less than the amount of nonfreezing water if some fraction of the water has altered thermodynamic properties but roughly normal translational and reorientational motion.

CONCLUSIONS

Our experimental results and conclusions may be summarized by the following statements.

(*a*) In fresh barnacle muscle fibers and in membrane-damaged fibers equilibrated with organic solutes, both water and solute protons are distinguishable in CW spectra of fibers confined in a capillary tube oriented perpendicularly to H_0. The chemical shift between water and methyl protons in the fibers is the same as in a simple solution, suggesting that there is no difference in shielding of any significant fraction of water molecules. Furthermore, linewidths of solute protons suggest they are not significantly bound to cell solids.

(*b*) FT spectra of fibers mounted on a Teflon block and oriented with their long axes at various angles to the magnetic field do indeed show shifts of the water peak and changes in linewidth. The methyl peak of TMAO, however, shows identical shifts, which can be reproduced by a capillary filled with pure water. We interpret these spectra as artifacts of gross susceptibility changes due to sample geometry.

(*c*) Analysis of the free induction and spin echo decay curves of various fibers indicates that, in addition to a very rapidly decaying signal from immobile protons on cell solids, ~5% of the remaining proton signal decays rapidly, with time constant $T_2 \sim 1$ ms. This fraction is assigned to mobile protons on cell solids. The remaining signal decays as a single exponential. In fibers devoid of organic solutes, its magnitude accounts for more than 95% of the fiber water; in fibers containing ~3% of protons as organic solutes, its magnitude is proportionately greater. This main decay fraction is taken as equivalent to cell water. No slow decaying fraction of extracellular water is seen in barnacle muscle.

(*d*) All relaxation processes are assumed to be due to dipolar coupling. The constancy of T_2 at different Larmor frequencies indicates that local field inhomogeneities do not contribute significantly to T_2. The identity in relaxation rates for 90°, τ, 90°$_{(\pi/2)}$(T_3) and 90°, τ, 180°$_{(\pi/2)}$ (T_2) pulse sequences further argues for the absence of residual dipolar splitting of > 180 Hz for motions with correlation times shorter than 1.8×10^{-3} s.

(*e*) The relaxation data are analyzed in terms of a two-compartment model of anisotropically tumbling bound and isotropically tumbling free water in rapid exchange. Equations are derived that permit calculation of the fraction of bound water (f) and hence of protein hydration (H_r): the frequency dependence of T_1 and $T_{1\rho}$ yield two dispersions. One, with mean correlation time of $\tau_b \simeq 10^{-8}$ s, is assigned to the anisotropic rotational motion of the bound water, with order parameter, $S_b \simeq 0.05$. The other, with $\tau_{ex} \simeq 10^{-5}$ s, is assigned to the exchange between bound and free fractions. A further low frequency dispersion with $\tau_c > 10^{-5}$ s appears to be present and is tentatively identified either with diffusion between variously oriented surface domains with the fiber or within proton exchange between water molecules.

(f) Despite three-fold differences in fiber hydration, values of τ_{ex}, τ_b, H_r, and S_b are constant within experimental error for variously treated fibers. We conclude that the effects of various cell solutes on macromolecular charge and its consequent effects on total fiber water content are not mediated through large changes in the nature of the water bound to macromolecular surfaces.

We gratefully acknowledge many fruitful discussions with Dr. Myer Bloom concerning the theoretical aspects of this work, and for the derivation of Eq. A5, given in Appendix B. We thank Laurie Nicol for technical assistance, and Dr. J.H. Davis for the use of his two exponential least squares computer program.

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APPENDIX A

Derivation of a Model for Water Protons in Barnacle Muscle

It is assumed that the relaxation of water protons in barnacle muscle is due to the time dependence of the dipolar Hamiltonian, $\mathcal{H}(t)$. $\mathcal{H}(t)$ is time-dependent owing to the various motions, translational and

rotational, of both a free fraction and a bound fraction of water, and also to the exchange of water molecules between the two fractions. Hence, it is possible to decompose $\mathcal{H}(t)$ into its time averaged and fluctuating components:

$$\mathcal{H}(t) = \langle \mathcal{H} \rangle + [\mathcal{H}(t) - \langle \mathcal{H} \rangle], \tag{A1}$$

where the first term is the mean value and the second, the fluctuations about the mean. We assume in this study that the correlation times τ_c for the various motions are all sufficiently short that $|\mathcal{H}|\tau_c \ll 1$, where $\mathcal{H}$ is expressed in units of angular frequency. Then, the line shape in the absence of complete motional narrowing is governed by the secular part of $\langle \mathcal{H} \rangle$ and the spin-lattice relaxation by the fluctuating term of Eq. A1.

For our two-site model we can write an Eq. A1 for each site; for the free water (a) which is assumed to tumble isotropically

$$\langle \mathcal{H} \rangle_a = 0, \tag{A2}$$

but for the bound water (b) which may be tumbling anisotropically

$$\langle \mathcal{H} \rangle_b = \mathcal{H}(0) S_b \neq 0. \tag{A3}$$

$\mathcal{H}(0)$ is the dipolar Hamiltonian at some arbitrary instant of time (i.e., in the absence of motion.) S_b for the secular part of $\langle \mathcal{H} \rangle_b$ is defined as

$$S_b = \left\langle \frac{3}{2} \cos^2 \theta - \tfrac{1}{2} \right\rangle, \tag{A4}$$

and is the order parameter (Saupe and Englert, 1963) of the water while bound; θ is the angle that the proton-proton vector makes with the static magnetic field, H_0, for the particular case when the symmetry axis (usually the normal) of the surface lies along H_0. The order parameter S_b can take values ranging between $+1$ and $-\frac{1}{2}$; for isotropic motion, $S_b = 0$. According to Eqs. A2 and A3, if the exchange between free and bound water fractions is slow compared with the rate of motion in either fraction, then this exchange will modulate $\mathcal{H}$ between $\langle \mathcal{H} \rangle_b$ and zero. This modulation will be an additional relaxation mechanism. If we assume that there is no correlation between water in the free and bound fractions, then it may readily be shown that the correlation function for our two-site model is

$$G(t) = f[(1-f)e^{-t/\tau_{ex}} + f][(\langle |\mathcal{H}_b(0)|^2 \rangle - \langle \mathcal{H} \rangle_b^2) g_b(t) + \langle \mathcal{H} \rangle_b^2] + (1-f)[f e^{-t/\tau_{ex}} + (1-f)][\langle |\mathcal{H}_a(0)|^2 \rangle g_a(t)]. \tag{A5}$$

(The derivation of this equation is given in Appendix B). In Eq. A5, f is the fraction of water that is bound; $\tau_{ex} = (W_{ab} + W_{ba})^{-1}$ is the time constant for exchange between the two sites were W_{ij} is the transition probability of a water molecule in site i going to site j; $g_a(t)$ and $g_b(t)$ are the reduced correlation functions for the free and bound fractions, and include those water molecules that start and end in the same site regardless of whether or not they spend time in the other site. The reduced correlation functions $g_a(t)$ and $g_b(t)$ are not necessarily exponential; however, in the absence of a better model we make the approximations

$$g_a(t) = e^{-t/\tau_a} \tag{A6}$$

$$g_b(t) = e^{-t/\tau_b}; \tag{A7}$$

where τ_a and τ_b are the correlation times of the free and bound water. Under conditions where all relaxation is due to proton-proton dipolar interactions (as assumed here), and providing that the molecular structure of water is constant for all fractions (a reasonable assumption), then the following

two terms from Eq. A5 become equivalent:

$$\langle |\mathcal{H}_b(0)|^2 \rangle = \langle |\mathcal{H}_a(0)|^2 \rangle \equiv \langle |\mathcal{H}(0)|^2 \rangle. \tag{A8}$$

The general theory of relaxation (Abragam, 1961) then gives the NMR relaxation rates in terms of the Fourier transform of $G(t)$. We shall assume (as have Diegel and Pintar, 1975) that τ_{ex} is much larger than τ_a or τ_b, and we then obtain

$$1/T_i = f(1 - S_b^2)\, 1/T_i(b) + (1 - f)\, 1/T_i(a) + f(1 - f)S_b^2\, 1/T_i(ex), \tag{A9}$$

where

$$1/T_i(j) = (K/2)\,[A\,J_j(\omega_0) + B\,J_j(2\omega_0) + C\,J_j(2\omega_1)], \tag{A10}$$

where

$$K = (3/10)\,(\gamma^4\hbar^2/r^6) + K_{\text{inter}} \tag{A11}$$

and

$$J_j(\omega) = 2\tau_j/1 + \omega^2\tau_j^2. \tag{A12}$$

In Eqs. A9–A12, T_i is T_1, $T_{1\rho}$, or T_2 and for T_1 we set $A = 1$, $B = 4$, and $C = 0$; for $T_{1\rho}$ and T_2 we set $A = 2.5$, $B = 1$, and $C = 1.5$; $\omega_1/\gamma = H_1$ is the strength of the spin-locking field in the $T_{1\rho}$ experiments, and for T_2 the value of ω_1 is 0; j labels the relaxation mechanism as that appropriate to the bound water (b), the free water (a), or the exchange (ex); γ is the proton gyromagnetic ratio; $\hbar$ is Planck's constant divided by 2π; r is the proton-proton distance in water; and ω_0 is the Larmor precession frequency. K_{inter} is the contribution to relaxation arising from intermolecular dipolar interactions; we shall use $K = 1.6 \times 10^{10}\text{s}^{-2}$ as obtained from the measured value of the second moment M_2 (Rabideau et al., 1968), where $M_2 = (3/2)K$ (Abragam, 1961). We have assumed that

$$\langle \mathcal{H} \rangle_b \ll \omega_1. \tag{A13}$$

That is, we have assumed that the weak collision limit is applicable for $T_{1\rho}$ (Ailion, 1971). We note that if there is isotropic motion in the bound water fraction, i.e., if $S_b = 0$, then Eq. A9 reduces to that obtained for the fast exchange limit by Zimmerman and Brittin (1957).

Whenever anisotropic motion exists in the bound fraction, then $\langle \mathcal{H} \rangle_b \neq 0$ and there is a time independent term $f^2\langle \mathcal{H} \rangle_b^2$ in Eq. A5. This time independent term corresponds to a residual Hamiltonian, $\langle \mathcal{H} \rangle_{av}$, time-averaged over those motions with correlation times τ_a, τ_b, and τ_{ex}. In a water proton-containing system, $\langle \mathcal{H} \rangle_{av}$ may be reduced to zero via an isotropic low frequency (lf) relaxation mechanism with correlation time τ_{lf}. We should also note, however, that any such low frequency motions will influence T_2 only when the inequality

$$\pi\Delta\nu\tau_{lf} \lesssim 1 \tag{A14}$$

is satisfied. If the low frequency mechanism is proton exchange between water molecules ($\tau_{lf} \equiv \tau_{pe} = 1.8 \times 10^{-3}$s at 25°C and pH7.0; Meiboom, 1961), then $\Delta\nu$ in Eq. A14 is the residual proton spectral splitting in Hertz that would be observed for the water in the absence of proton exchange. If the inequality holds, then exchange of protons between different water molecules will reduce the values of $\langle \mathcal{H} \rangle_{av}$ and $\Delta\nu$ to zero; i.e., as long as $\Delta\nu < 180$ Hz, the residual dipolar splitting will be motionally narrowed to a single Lorentzian line of full width $= (\pi T_2)^{-1}$ at half maximum.

As long as the correlation time for these low frequency motions τ_{lf} is much greater than τ_a, τ_b, and τ_{ex}, then all except the $f^2\langle \mathcal{H} \rangle_b^2$ term in Eq. A5 will decay to zero before these low frequency motions affect the correlation function. At long times we can write the correlation function as

$$G'(t) = G(t)\, g_{lf}(t)$$

$$= f^2\, \mathcal{H}(0)^2\, S_b^2\, g_{lf}(t), \tag{A15}$$

where $g_{lf}(t)$ is the reduced correlation function corresponding to the low frequency motion; as before we assume

$$g_{lf}(t) = e^{-t/\tau_{lf}}. \tag{A16}$$

Hence, we must add another term, $1/T_2'$, to Eq. A9 such that the observed value of $1/T_2$ is now given by

$$1/T_2 = 1/T_2 \text{ (from Eq. A9)} + 1/T_2', \tag{A17}$$

where

$$1/T_2' = (3/2)\, f^2\, S_b^2\, K\, \tau_{lf}. \tag{A18}$$

APPENDIX B

The calculation presented here is due to Myer Bloom, Physics Department, University of British Columbia.

We wish to derive the generalized correlation function appropriate to a two-site system where the time-averaged dipolar Hamiltonian $\langle \mathcal{H} \rangle$ is not necessarily zero in either site, and where there is exchange between sites. Consider a system with two sites, a and b, where the number of molecules in each site is N_a, N_b and

$$N_a + N_b = N. \tag{B1}$$

N is the total number of molecules and is constant. The equilibrium probability P_i of a molecule being in fraction i is defined by

$$P_i = N_i/N \text{ (at equilibrium)}. \tag{B2}$$

The dipolar Hamiltonian in each site $\mathcal{H}_i(t)$ can be expressed in terms of its time-averaged and fluctuating components

$$\mathcal{H}_i(t) = \langle \mathcal{H} \rangle_i - [\mathcal{H}_i(t) - \langle \mathcal{H} \rangle_i]. \tag{B3}$$

The correlation function $G_{ij}(t)$ for a molecule that starts at time $t=0$ in site i and ends up at time t in site j is

$$G_{ij}(t) = \langle \mathcal{H}_i(0)\, \mathcal{H}_j(t) \rangle. \tag{B4}$$

If $i = j$ Eq. B4 can be written

$$G_{ii}(t) = \langle \mathcal{H}_i(0)\, \mathcal{H}_i(t) \rangle$$

$$= [\langle |\mathcal{H}_i(0)|^2 \rangle - \langle \mathcal{H} \rangle_i^2]\, g_i(t) + \langle \mathcal{H}_i(0) \rangle^2, \tag{B5}$$

where we have assumed that we can write a reduced correlation function $g_i(t)$ to include all molecules that are in state i at time 0 and again at time t, regardless of the number of visits to site j. We now assume that there is no correlation between different sites i and j, that is

$$G_{ij}(t) = \langle \mathcal{H}_i(0)\mathcal{H}_j(t) \rangle = \langle \mathcal{H}_i(0) \rangle\, \langle \mathcal{H}_j(t) \rangle, \qquad i \neq j. \tag{B6}$$

We define W_{ij} as the transition probability per unit time of a molecule going from state i to j, and $P_{ij}(t)$

as the conditional probability of a molecule being in state j at time t given that it is initially in state i. Then the general correlation function for the system is

$$G(t) = \sum_{i=a,b} \sum_{j=a,b} P_i P_{ij}(\mathrm{t})\, G_{ij}(\mathrm{t}). \tag{B7}$$

The rate of exchange between states is

$$\mathrm{d}N_i/\mathrm{d}t = -N_i W_{ij} + N_j W_{ji} \qquad i \neq j \tag{B8}$$

or

$$\mathrm{d}N_i/\mathrm{d}t + (W_{ij} + W_{ji})N_i = W_{ji}N. \qquad i \neq j \tag{B9}$$

The general solution to Eq. B9 is

$$N_i = A\, \mathrm{e}^{-t/\tau} + W_{ji}\, \tau N, \tag{B10}$$

where A is a constant and where

$$1/\tau = W_{ij} + W_{ji}. \tag{B11}$$

To calculate A we use as initial condition at $t = 0$

$$N_i = N \tag{B12}$$

and obtain from Eq. B10

$$A = W_{ij}\, \tau N. \tag{B13}$$

Hence, we can calculate $P_{ii}(t)$ and $P_{ij}(t)$ as

$$\begin{aligned} P_{ii}(t) &= N_i(t)/N \\ &= W_{ij}\, \tau \mathrm{e}^{-t/\tau} + W_{ji}\tau \end{aligned} \tag{B14}$$

and

$$\begin{aligned} P_{ij}(t) &= 1 - P_{ii}(\mathrm{t}) \\ &= W_{ij}\tau(1 - \mathrm{e}^{-t/\tau}). \qquad i \neq j \end{aligned} \tag{B15}$$

As a check, we note the limiting cases at $t = 0$

$$P_{ii}(0) = 1 \tag{B16}$$

$$P_{ij}(0) = 0 \qquad i \neq j \tag{B17}$$

and at $t = \infty$

$$P_i = P_{ii}(\infty) = W_{ji}\tau = \frac{W_{ji}}{W_{ij} + W_{ji}} \qquad i \neq j \tag{B18}$$

$$P_j = P_{ij}(\infty) = W_{ij}\tau = \frac{W_{ij}}{W_{ij} + W_{ji}} \qquad i \neq j \tag{B19}$$

Note detailed balance requires, as obtained, that

$$P_i/P_j = W_{ji}/W_{ij}. \tag{B20}$$

The above is a general solution to our problem. We now examine the special case of interest where we set

$$\langle \mathcal{H}_a \rangle = 0 \tag{B21}$$

$$\langle \mathcal{H}_b \rangle = \langle \mathcal{H} \rangle_b. \tag{B22}$$

Substituting Eqs. B21 and B22 into Eq. B6, and this in turn into Eq. B7 we get

$$G(t) = P_a P_{aa}(t) \langle \mathcal{H}_a(0) \mathcal{H}_a(t) \rangle + P_b P_{bb}(t) \langle \mathcal{H}_b(0) \mathcal{H}_b(t) \rangle. \tag{B23}$$

Using Eqs. B5, B14, and B23 we get

$$\begin{aligned} G(t) = {} & P_a [W_{ab}\tau \, e^{-t/\tau} + W_{ba}\tau] \langle |\mathcal{H}_a(0)|^2 \rangle g_a(t) \\ & + P_b [W_{ba}\tau e^{-t/\tau} + W_{ab}\tau] [\langle |\mathcal{H}_b(0)|^2 \rangle - (\langle \mathcal{H} \rangle_b^2) g_b(t) + \langle \mathcal{H}_b(0) \rangle^2]. \end{aligned} \tag{B24}$$

Using Eq. B18 or B19, Eq. B24 becomes

$$\begin{aligned} G(t) = {} & P_a [P_b e^{-t/\tau} + P_a] \langle |\mathcal{H}_a(0)|^2 \rangle g_a(t) \\ & + P_b [P_a e^{-t/\tau} + P_b] [(\langle |\mathcal{H}_b(0)|^2 \rangle - \langle \mathcal{H} \rangle_b^2) g_b(t) + \langle \mathcal{H} \rangle_b^2], \end{aligned} \tag{B25}$$

which is our desired result.

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