WATER IN BARNACLE MUSCLE

IV. Factors Contributing to Reduced Self-Diffusion

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ABSTRACT The relative self-diffusion coefficients, $\mathcal{D}/\mathcal{D}_0$, of water in various solutions, in fresh barnacle muscle fibers, and in membrane-damaged fibers equilibrated with several media have been estimated from NMR relaxation rates in the presence of applied field gradients. A model has been developed to account for the contributions to the observed reduction in $\mathcal{D}/\mathcal{D}_0$ from small organic solutes, and from the hydration and obstruction effects of both soluble macromolecules and myofilament proteins. Intracellular ions do not affect $\mathcal{D}/\mathcal{D}_0$, but all tested organic solutes do. Solute effects are additive. When artificially combined in the proportions found in barnacle muscle ultracentrifugate (measured $\mathcal{D}/\mathcal{D}_0 = 0.77$), organic acids, small nitrogenous solutes, and proteins give $\mathcal{D}/\mathcal{D}_0 = 0.77$. After correcting the $\mathcal{D}/\mathcal{D}_0$ measured in fibers for this value, we calculate the myofilament hydration, H_m, in fresh muscle to be 0.65 g H_2O/g macromolecule. Only in membrane-damaged fibers, highly swollen by salt-rich media, was this significantly increased. Because our earlier NMR relaxation measurements indicate only 0.07 g H_2O bound/g myofilament protein, we conclude that the "hydration" water measured by reduction of $\mathcal{D}/\mathcal{D}_0$ cannot be described by stationary layers of water molecules; instead, we propose that nonpolar groups on the proteins cause extensive, hydrophobically-induced interactions among a large fraction of solvent molecules, slowing their translational motion.

INTRODUCTION

In this paper, the last of a series of studies on water in barnacle muscle fibers, we report values of the water self-diffusion coefficient in fresh and variously treated fibers that complement our earlier results. As noted in the third of these papers (Burnell et al., 1981, p. 13), NMR measurements of water associated with various macromolecular systems regularly yield reductions, relative to pure water, of about fivefold for the spin-lattice relaxation time (T_1) and of up to fiftyfold for the spin-spin relaxation time (T_2); in addition, the relative self-diffusion coefficient of water, $\mathcal{D}$, in barnacle muscle fibers relative to that in pure water, $\mathcal{D}_0$, is reduced to 0.5 or 0.6.

In muscle cells, several factors may contribute to this observed reduction of the self-diffusion coefficient: (*a*) rigid solids may physically obstruct free motion; (*b*) water that is bound[1] to such solids may have reduced translational motion; (*c*) water bound to dissolved macromolecules may have reduced translational motion; (*d*) water associated with small solute molecules may have reduced translational motion.

In this paper, we analyze the various contributions of these factors, using information obtained earlier about the dry weight components of fresh barnacle fibers (Clark and Hinke, 1981). Only about half the dry weight is insoluble matter (mainly myofilament protein), 23% of the total dry weight is soluble high molecular weight solids, 23% is small organic solutes, and 3% is inorganic ions. In our experiments, we have measured the rate of decay of spin-echo signals in the presence of known magnetic field gradients to estimate the average value of the self-diffusion coefficient of water in fresh fibers, in experimentally treated fibers, in isolated sarcoplasmic fluid, and in artificial mixtures containing various combinations of cell solutes. As we shall show, only the ions do not contribute to the reduced self-diffusion coefficient; both soluble macromolecules and small solutes found in the sarcoplasmic fluid make important contributions to reduced translational motion of muscle cell water. This analysis permits us to calculate the fraction of fiber water that has reduced motion owing to its association with macromolecules in the cell, particularly the myofilament proteins.

As we have shown earlier, when membrane-damaged fibers are equilibrated with a solution containing only inorganic ions, they swell greatly, in proportion to the ionic strength; but if naturally occurring organic solutes (amino

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[1]The term "bound water" has been given many meanings. Throughout this paper, it signifies that water that has measurably restricted motions owing to its association with slowly moving or stationary molecules, mainly proteins. It does not imply any particular thermodynamic state. Similarly, the unbound or "free water" described in this simple model is not necessarily thermodynamically uniform, and must be taken only as water without demonstrable motional restrictions.

acids, trimethylamine oxide [TMAO], or glycerol) are added as well, at concentrations commensurate with those found in living cells, this swelling does not occur. Thus, normal cell volume depends partly upon intracellular solute composition (Clark et al., 1981). These observations raised the question whether the salt-induced swelling is correlated with changes in the amount of water bound to the myofilament proteins. This question has already been approached by utilizing NMR relaxation techniques (Burnell et al., 1981). Briefly, we have found that the fiber water protons exhibit a single exponential free induction decay (FID) regardless of treatment or total fiber water content, and the measured relaxation rate varies inversely with fiber water content. This result argues that the additional water present in swollen fibers is not rotationally restricted. Our T_1 and $T_{1\rho}$ measurements indicated that the relaxation mechanisms in variously treated fibers are indistinguishable. The model we have assumed to explain all of our results is one in which there is a small fraction of bound water associated with the macromolecular surfaces that has anisotropic rotational motion; however, it exchanges rapidly ($\tau_{ex} \simeq 10^{-5}$ s) with the remaining free water in the fiber. The weight of water bound per weight of macromolecule is approximately constant at a ratio of 0.1 to 1 regardless of treatment.

Here we report independent estimates of the amount of water bound to macromolecules in fresh and treated fibers, calculated from NMR measurements of the reduction in the self-diffusion coefficient, appropriately corrected for the contribution from soluble components. On comparing these two approaches, we find that self-diffusion measurements in all fibers give an estimate of water bound to macromolecules that is an order of magnitude higher than that obtained by relaxation measurements. Possible reasons for these differences are discussed.

MATERIAL AND METHODS

Samples

The composition of the various solutions investigated is given in Tables I and II. Table I contains solutions used to equilibrate membrane-damaged fibers and several modifications thereof, as well as glycerol-containing solutions. (Glycerol was included because it is an important solute in frost-resistant insects and plants, and was found to behave like other organic solutes in preventing salt-induced swelling of membrane-damaged fibers [Clark et al., 1981].)

Table II contains solutions that comprise various combinations of barnacle muscle solutes. These include: (*a*) inorganic salt solutions, where the counterion for potassium is propionate, a model solute for organic acids and phosphorylated sugars that probably make up most of the 100 mosmol of unidentified organic solutes in the fiber (Clark and Hinke, 1981); (*b*) mixtures of small nitrogenous solutes in the proportions found in barnacle muscle; (*c*) solutions of bovine serum albumin (BSA), used as a model for the soluble macromolecules in barnacle muscle; (*d*) various combinations of the above. The source of each chemical is given in the table. Concentrations are molal, and the mole fraction of water in each solution is given, together with the measured pH and milliosmolarity. The latter was measured on a Wescor vapor pressure osmometer (Wescor Inc., Logan, UT), model 5100B, calibrated from 0 to 1,000 mOsM.

Single scutal depressor muscle fibers dissected from the giant barna-

TABLE I
COMPOSITION OF SOLUTIONS AND RELATIVE SELF-DIFFUSION COEFFICIENTS*

Solutions	Na	K	Mg	Cl	Propionate	TRIS	EGTA	TMAO	Glycerol	$\mathcal{D}/\mathcal{D}_0$ 1A	2A	3A	Average ± SE
Salt													
TOO	0	0	0	0	11.5	13.5	5	0	0	not measured			
TMO	50	150	10	220	11.5	13.5	5	0	0	1.043	0.993		1.018 ± 0.025
											1.056	1.079	1.068 ± 0.012
TMO/170	50	150	10	50	181.5	13.5	5	0	0	0.979	0.929		0.954 ± 0.025
Organics only													
TO5	0	0	0	0	11.5	13.5	5	500	0		0.875	0.842	0.859 ± 0.017
TO6	0	0	0	0	11.5	13.5	5	600	0	not measured			
T.25	0	0	0	0	0	0	0	250	0	0.950	0.910		0.930 ± 0.020
T.50	0	0	0	0	0	0	0	500	0	0.862	0.830		0.846 ± 0.016
T.75	0	0	0	0	0	0	0	750	0	0.814	0.749		0.782 ± 0.033
T1.00	0	0	0	0	0	0	0	1,000	0	0.715	0.678		0.697 ± 0.019
Salt + organics													
TM6	50	150	10	220	11.5	13.5	5	600	0	not measured			
TM5	50	150	10	220	11.5	13.5	5	500	0	0.857	0.831		0.844 ± 0.013
											0.855	0.920	0.888 ± 0.032
TM5-P/170	50	150	10	50	181.5	13.5	5	500	0	0.805	0.756		0.781 ± 0.025
G.2	50	150	10	220	11.5	13.5	5	0	200	0.980	0.939		0.960 ± 0.021
G.6	50	150	10	220	11.5	13.5	5	0	600	0.893	0.856		0.875 ± 0.019

*Errors in slopes fitted by linear least-squares regression always <2%; all concentrations in millimoles per liter.

TABLE II
COMPOSITION OF SOLUTIONS MIMICKING CELL SOLUTES*

Solutions	Component																	
	K (KOH)	Propionic acid	NaCl	GLY‡	ASN	PRO	ARG	TAU	ALA	VAL	GLU	BET	TMAO	BSA	$\bar{X}_{H_2O}$§	pH	mOsM (measured)	mOsM‖ (theoretical)
Salt																		
S-1 (same as in muscle)	150	150	35	—	—	—	—	—	—	—	—	—	—	—	0.994	5.70	294	370
S-2 (~1,000 mOsM)	405	405	95	—	—	—	—	—	—	—	—	—	—	—	0.982	5.60	78	1,000
Organic																		
O-1 (same as in muscle)	—	—	—	300	50	50	35	25	20	10	10	50	50	—	0.989	5.30	604	600
O-2 (~1,000 mOsM)	—	—	—	500	83	83	58	42	33	17	17	83	83	—	0.982	5.40	965	999
Protein																		
P-1 (4 g/100 g H_2O)	—	—	—	—	—	—	—	—	—	—	—	—	—	0.6	1.000	5.00	not	1
P-2 (8 g/100 g H_2O)	—	—	—	—	—	—	—	—	—	—	—	—	—	1.2	1.000	5.20	mea-	2
P-3 (16 g/100 g H_2O)	—	—	—	—	—	—	—	—	—	—	—	—	—	2.4	1.000	5.15	sured	3
Combinations																		
S/P [(S-1) + (P-2)]	150	150	35	—	—	—	—	—	—	—	—	—	—	1.2	0.993	5.70	298	372
O/S [(O-1) + (S-1)]	150	150	35	300	50	50	35	25	20	10	10	50	50	—	0.983	5.60	864	970
O/P [(O-2) + (P-2)]	—	—	—	300	50	50	35	25	20	10	10	50	50	1.2	0.989	5.23	592	602
S/O/P [(S-1) + (O-1) + (P-2)]	150	150	35	300	50	50	35	25	20	10	10	50	50	1.2	0.983	5.60	875	972
Cell ultracentrifugate¶ 8 g macromolecule / 100 g H_2O	170	(149)	35	279	47	47	34	27	19	8	9	41	41	~1.2			990	

*Concentrations in millimoles per kilogram water.

‡Abbreviations, GLY, glycine; ASN, asparagine; PRO, proline; ARG, arginine; TAU, taurine; ALA, alanine; VAL, valine, GLU, glutamic acid; BET, glycine betaine; TMAO, trimethylamine-*N*-oxide; BSA, bovine serum albumin. BSA concentrations based on a molecular mass of 6.7×10^4 Daltons (JACS, 68, 459, 1946); the sources for the chemicals are as follows: KOH, propionic acid, and NaCl, J. T. Baker Chemical Co., Phillipsburg, NJ; GLY, ARG, TAU, VAL, BET, and BSA, Sigma Chemical Co., St. Louis, MO; ASN and ALA, Nutritional Biochemical Corp, Cleveland, OH; PRO, Eastman Kodak, Co, Rochester, NY; GLU, Fisher Scientific Co., Pittsburgh, PA; TMAO, Aldrich Chemical Co, Inc, Milwaukee, WI.

§X_{H_2O} is the mole fraction of water in each solution.

‖mOsM (theoretical) is the theoretical osmolarity of the solution assuming all osmotic coefficients = 1.

¶Approximate composition only; total organic acids and phosphorylated intermediates estimated at 149 mmol/kg (listed under propionic acid). For complete composition, see Clark and Hinke (1981).

cle, *Balanus nubilus*, were used either fresh or following membrane disruption by detergent and equilibration with various solutions as previously described (Clark et al., 1981). Each 7-mm NMR tube contained two to five thoroughly blotted fibers which, with one exception, had been minced on a dry glass surface. Fibers were kept at 0°C until measurements were made. For each diffusion measurement, the water content was determined on five similarly treated companion fibers. An ultracentrifugate of fresh fibers was prepared as previously described (Clark and Hinke, 1981) and its T_1, T_2, and self-diffusion coefficient were measured the same day.

NMR Measurements

The self-diffusion coefficient of water in the samples was measured by following the rate of decay of the echo at time 2τ of a 90°, τ, $180°_{(\pi/2)}$ NMR pulse sequence in the presence of a field gradient, where the $\pi/2$ subscript indicates that the phase of the radio frequency carrier of the second pulse is shifted by $\pi/2$ relative to the first pulse. The relationship (Abragam, 1961) is given by

$$A(2\tau) = A(0) \exp(-2\tau/T_2 - 2/3\,\gamma^2\,\mathcal{D}\,\tau^3 G^2) \qquad (1)$$

where $A(2\tau)$ is the height of the echo, T_2 is the spin-spin relaxation time, γ is the gyromagnetic ratio, $\mathcal{D}$ is the self-diffusion coefficient, and G is the field gradient in gauss (G) per centimeter. T_2 was evaluated using either the 90°, τ, $180°_{(\pi/2)}$ sequence, or the CPMG sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958) corrected for base-line drift by the procedure described by Hughes (1977) and Hughes and Lindblom (1974, 1977). $\mathcal{D}$ was obtained from the slope of a plot of $\ln A(2\tau) + 2\tau/T_2$ vs. τ^3.

All measurements were made on a Bruker pulse spectrometer (Bruker Instruments, Inc., Billerica, MA, model BKR 322-S). During each experiment, the temperature of the probe was maintained within ±0.5° K. Depending on ambient air temperatures and field gradient strengths, probe temperatures ranged from 294° to 298°K. Larmor frequencies also differed with the experiment, in the range from 20 to 45 MHz. Field gradients were applied in the range of 10 to 43 G/cm; in most cases measurements at two gradients were made on each sample. Signal amplitudes were recorded either on a storage oscilloscope (Tektronix, Beaverton, OR) equipped with a polaroid camera (and then were measured manually) or on a Nicolet 1090 AR "Explorer" signal amplitude computer (Nicolet Instrument Corp., Madison, WI) interfaced to an Intel 8080A microprocessor. Both recorders responded linearly to signal intensity. Spacing between pulse sequences was always greater than ten times T_1 of the sample. Increments of τ were ~0.4 ms during the steep part of the decay curve and plots of $\ln A(2\tau) + 2\tau/T_2$ vs. τ^3 were always linear after the first 6–8 ms (Fig. 1). By 2τ~15–30 ms, depending on the sample and gradient used, the signal had decayed to <10% of $A(0)$ and unfavorable signal-to-noise usually precluded further readings.

Because the probe temperature varied slightly with each experiment, all results are expressed as a ratio, $\mathcal{D}/\mathcal{D}_o$, the diffusion coefficient of the sample relative to that of water under identical conditions.

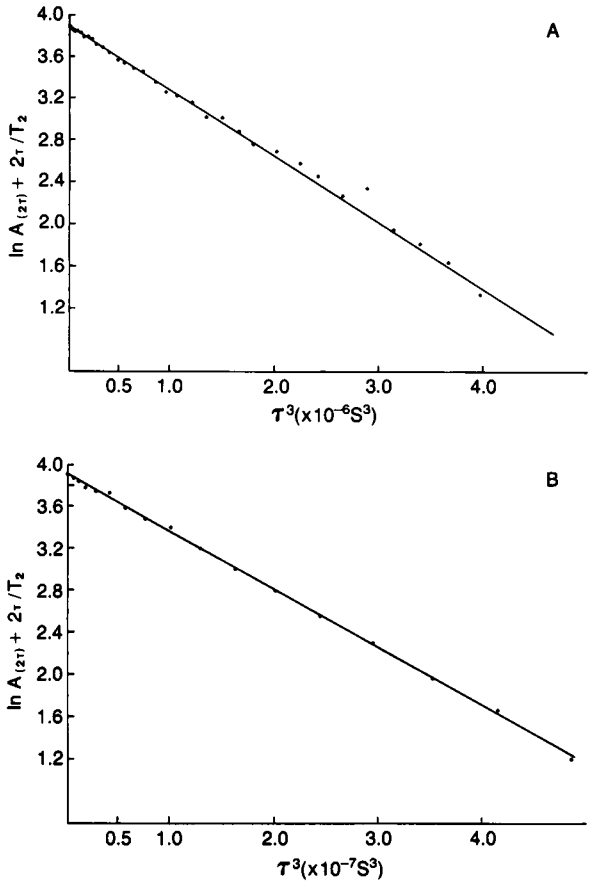

FIGURE 1 Estimation of self-diffusion coefficient of water, $\mathcal{D}$, by spin-echo decay in the presence of a steady-state magnetic field gradient; typical examples of linearity of $\ln A_{(2\tau)} + 2\tau/T_2$ vs. τ^3 (in s^3). *A*, fresh fibers. minced, in gradient of 9.87 gauss · cm^{-1}, $T_2 = 0.043$ s, $\mathcal{D} = 1.310 \pm 0.013\ 10^{-5}$ $cm^2 \cdot s^{-1}$. *B*, salt-treated (TMO) fiber, minced, in gradient of 24.10 gauss · cm^{-1}, $T_2 = 0.170$ s, $\mathcal{D} = 1.993 \pm 0.011\ 10^{-5}$ $cm^2 \cdot s^{-1}$.

METHOD OF ANALYSIS

Argument

For water molecules diffusing in a barrier-free system, the root-mean-square displacement of a water molecule in a specified direction, $\langle (\Delta X)^2 \rangle^{1/2}$, that occurs during time, t, is related to the self-diffusion coefficient, $\mathcal{D}$, by the Einstein relationship:

$$\langle (\Delta X)^2 \rangle = 2\mathcal{D}t. \tag{2}$$

In living cells, however, there exist both barriers that halt and obstructions that restrict free motion. Depending on the spacing of these restrictions, the measured value of $\mathcal{D}$ can be less than the true value for the cell fluid in the absence of such restrictions. In theory, if the time 2τ can be made short enough (much less than the root-mean-square time for a molecule to diffuse between barriers or obstructions [Wayne and Cotts, 1966; Woessner, 1963]), NMR pulse techniques should permit a direct measurement of the true value of $\mathcal{D}$ in the cell fluid.

If diffusion barriers are present and sufficiently close together, the echoes no longer decay as τ^3 (Wayne and Cotts, 1966 and references therein; Neuman, 1974). In all our experiments the echo decays were linear in τ^3 to $2\tau \sim 30$ ms (Fig. 1); hence our experiments are unlikely to have been affected by the presence of barriers that are impermeable to water. (We note that the minced fiber fragments were a few mm long, and hence mincing was unlikely to have influenced apparent barriers. Furthermore, unminced fibers gave similar results.) Although Hansen (1971) reported barriers to diffusion in rat muscle and brain tissue, more recently Rorschach et al. (1973), using arguments for rat gastrocnemius muscle barriers that were proposed by Chang et al. (1973), have concluded that neither the plasma membrane, the Z-line structure, nor the sarcoplasmic reticulum reduces the value of $\mathcal{D}$ in that tissue by acting as a barrier to diffusion. Because the spacing of potential barriers in barnacle muscle is generally greater than that in rat muscle (Hoyle et al., 1973) we are not surprised to observe no evidence of barriers to diffusion in our experiments.

The presence of obstructions (myofilaments) about which the water molecules must diffuse will lower the value of $\mathcal{D}$ for the cell water if the spacing between filaments is $< [4\mathcal{D}(2\tau)]^{1/2}$ cm. This condition is satisfied by the myofilaments in the barnacle muscle fibers used in our experiments. A method for estimating this obstruction effect was presented by Wang (1954) for an ovalbumin solution, and has been applied by others to the analysis of diffusion coefficients of ions in polyelectrolyte solutions (Rice and Nagasawa, 1961), of various substances in gels (Lauffer, 1961), and of water in muscle (Caillé and Hinke, 1974).

Applicability of the Wang Equation in This Study

Wang (1954) suggested that the value of $\mathcal{D}$ obtained for water in pure water/protein solutions incorporates two components: an obstruction component, due to the basically immobile protein molecules, and a slowing down of diffusion owing to binding of some fraction of the water to macromolecular surfaces. These effects are related by the following expression (Wang, 1954)

$$\mathcal{D} = \mathcal{D}_0 (1 - \bar{\alpha}\phi)(1 - f). \tag{3}$$

The term $(1 - \bar{\alpha}\phi)$ is the obstruction contribution to diffusion, where ϕ is the volume fraction of the hydrated protein molecules and $\bar{\alpha}$ is a constant defined by the shape and orientation of the molecules relative to the axis of diffusion. The term $(1 - f)$ is the fraction of water free to diffuse, whence (f) is the fraction of water immobilized on the surface of the protein. This bound water is assumed to exchange rapidly with the free water compared with the timescale of the measurement.

The general validity of Wang's theory has been criticized by several authors (Lauffer, 1961; Cleveland et al., 1976; and Rorschach and Hazlewood).[2] When it is assumed that the macromolecules diffuse much more slowly than does the water, and that they are infinitely dilute, the contributions to water self-diffusion proposed by Wang are more correctly approximated by the following relation:

$$\mathcal{D} = \mathcal{D}_0 (1 - \bar{\alpha}\phi)(1 - f)/(1 - \phi). \tag{4}$$

Eq. 4 differs from that of Wang (1954) by the factor $(1 - \phi)$ in the denominator. The reasons for including this term are discussed in the Appendix.

The parameters f and ϕ both depend on the same variable, H, defined by Wang as the grams of bound water per gram of anhydrous protein. These parameters can be written:

$$\phi = \frac{\bar{V}_p + H/d_0}{\bar{V}_p + (1/d_0)\left(\frac{1 - w}{w}\right)} \tag{5}$$

$$f = 1 - \left(\frac{w}{1 - w}\right)H \tag{6}$$

where $\bar{V}_p$ is the apparent specific volume of anhydrous protein in its aqueous solution, d_0 is the density of pure water, taken as 1.0, and w is the weight fraction of anhydrous protein in solution. As shown by Wang, the

[2]Rorschach, H. E., and C. G. Hazlewood. Unpublished manuscript.

values of $\bar{\alpha}$ and hence of the hydration, H, are relatively insensitive to errors in the assumed shape of a protein that is a prolate ellipsoid.

Use of the Modified Wang Equation for Diffusion in Nonoriented Muscle Fibers

Fresh muscle fibers or membrane-damaged fibers equilibrated with various solutions contain not only water in the aqueous phase but also solutes which vary in composition from one preparation to the next. As will be shown below, the self-diffusion coefficient of water is markedly reduced in solutions containing large amounts of organic solutes comparable to those found in living invertebrate muscle. Thus, to use Eq. 4 to determine the amount of water bound to myofilament proteins, it is necessary to discount that fraction of the reduction in $\mathcal{D}$ in a fiber that is due to the small solutes present. In place of $\mathcal{D}_0$ in Eq. 4 we therefore use $\mathcal{D}'$, the self-diffusion coefficient of water in the isolated aqueous phase. For membrane-damaged fibers that are assumed to contain no soluble proteins Eqs. 4–6 become:

$$\mathcal{D} = \mathcal{D}_0 \frac{(1 - \bar{\alpha}_m\phi_m)(1 - f_m)}{(1 - \phi_m)} \cdot \mathcal{D}/\mathcal{D}_0 = \mathcal{D}' \frac{(1 - \bar{\alpha}_m\phi_m)(1 - f_m)}{(1 - \phi_m)} \quad (7)$$

and

$$\phi_m = \frac{\bar{V}_m + H_m/d_0}{\bar{V}_m + (1/d_0)\left(\frac{1 - u - v}{v}\right) + \bar{V}_s\left(\frac{u}{v}\right)} \quad (8)$$

$$f_m = \left(\frac{v}{1 - u - v}\right)H_m \quad (9)$$

where $\mathcal{D}'/\mathcal{D}_0$ accounts for the average reduction in translational motion of sample water relative to pure water, u is the weight fraction of ions and small organic solutes in solution, $\bar{V}_s$ and $\bar{V}_m$ are the partial specific volume of the small solutes and myofilaments respectively, v is the weight fraction of anhydrous myofilament in the system, and the subscript m refers to myofilament. Hence, $[(1-u-v)/v]$ is grams water per gram insoluble (myofilament) dry weight and is calculated for the treated fibers by assuming that solutes have equal concentrations in the equilibration fluid and fiber water, an approximation that is reasonably accurate for this purpose (Clark et al., 1981).

Fresh muscle contains soluble proteins in addition to myofilaments, small solutes and water. Hence, Eqs. 4–6 must be modified as follows, Eq. 10 showing the modification for Eq. 4, Eqs. 11 and 12 for Eq. 5 solved for soluble protein and myofilament, respectively, and Eq. 13 showing the modification for Eq. 6:

$$\mathcal{D} = \mathcal{D}_0 \frac{(1 - \bar{\alpha}_p\phi_p - \bar{\alpha}_m\phi_m)(1 - f_p - f_m)}{(1 - \phi_p - \phi_m)} \cdot \mathcal{D}'/\mathcal{D}_0 \quad (10)$$

and

$$\phi_p = \frac{\bar{V}_p + H_p/d_0}{\bar{V}_p + (1/d_0)\left(\frac{1 - u - v - w}{w}\right) + \bar{V}_m(v/m) + \bar{V}_s(u/w)} \quad (11)$$

$$\phi_m = \frac{\bar{V}_m + H_m/d_0}{\bar{V}_m + (1/d_0)\left(\frac{1 - u - v - w}{w}\right) + \bar{V}_p(w/v) + \bar{V}_s(u/v)} \quad (12)$$

$$f_p + f_m = \left(\frac{w}{1 - u - v - w}\right)H_p + \left(\frac{v}{1 - u - v - w}\right)H_m. \quad (13)$$

The subscript p refers to soluble protein and m to myofilament; w is the weight fraction of anhydrous soluble protein. For fresh fibers, the ratio $(1-u-v-w/w)$ has been determined from the known water and insoluble dry weight content, as previously analyzed (Clark and Hinke, 1981, Table III).

To calculate H_m from Eqs. 7–9, values of $\bar{\alpha}_m$, $\bar{V}_m$, and $\bar{V}_s$ must be assigned. (For Eqs. 10–13, values of H_p, $\bar{\alpha}_p$, and $\bar{V}_p$ must also be assigned; these are considered below, when $\mathcal{D}/\mathcal{D}_0$ of protein solutions is discussed.) A value of $\bar{V}_m$ for myosin of 0.728 has been reported by Holtzer and Lowey (1956, 1959). It is not at all clear that $\bar{V}_m$ will remain constant with treatments, however. The question of $\bar{\alpha}_m$ is considered next.

The modified Wang model (Eqs. 4, 7, 10) for the effect of obstructions in the analysis of diffusion of water in muscle fibers assumes an infinitely dilute solution. As pointed out by Rorschach et al. (1973) and Cleveland et al. (1976), boundary conditions appropriate for the finite concentration of macromolecules found in muscle should be used. The myofilaments, the major macromolecules in muscle, can be modeled as a hexagonal array of extremely elongated prolate ellipsoids or cylindrical rods. An approximate calculation for diffusion perpendicular to the major axis of such rods can be performed by applying boundary conditions at a limited number of points on the hexagonal symmetry surface. When this is done, the shape factor $\alpha_\perp = 2$ appropriate for perpendicular diffusion should be multiplied by a factor of the order of $(1 + \phi_m)^{-1}$. Such a correction agrees qualitatively with the result of Cleveland et al. (1976). For diffusion parallel to the major axis of such rods, $\alpha_\parallel = 1$. In calculating $\bar{\alpha}_m$, the average shape factor for myofilament proteins when diffusion occurs in three dimensions, we have omitted the correction $(1 + \phi_m)^{-1}$ of $\alpha_\perp$. Because $\bar{\alpha}_m$ is already multiplied by ϕ_m in Eq. 7, this correction is second order in ϕ_m. Furthermore, this omission is to some extent cancelled since $\alpha_\parallel$ is somewhat greater than one, owing to the projections of the myosin heads and C-proteins from the myofilament surfaces. We have chosen, as a compromise, to use in Eqs. 7 and 10 a value of $\bar{\alpha}_m = 1/3\,\alpha_\parallel + 2/3\,\alpha_\perp = 1.667$, appropriate to an infinitely dilute solution of smooth elongate rods. For $\bar{\alpha}_p$ of soluble proteins, which are present in muscle in much smaller amounts than are myofilament proteins (Clark and Hinke, 1981), Eq. 10 should represent an adequate approximation for our purpose. Because equations appropriate for concentrated solutions will predict larger values of hydration (Shantz and Lauffer, 1962), our use of Eqs. 4–6, 7–9, and 10–13 yields lower limits for macromolecular hydration.

As noted above, the effect of dissolved solutes on the average translational motion of the water molecules has been taken into account by introducing $\mathcal{D}'$. The contribution of these solutes to the solution volume affects ϕ_p and ϕ_m by the terms $\bar{V}_s(u/w)$ and $\bar{V}_s(u/v)$; however, these contributions are of much smaller magnitude than the other terms in the denominators, and little error is introduced by setting $\bar{V}_s = 0$.

RESULTS AND DISCUSSION

In reporting the relative self-diffusion coefficients of solutions and fibers, we have averaged the values obtained with different strength field gradients. For fibers, we have also averaged values for replicate samples run on different days. There were no significant differences on measurements of the same sample, and we have no reason to discard any measurements.

$\mathcal{D}/\mathcal{D}_0$ of Equilibrating Solutions

The relative self-diffusion coefficients of the equilibrating solutions are given in Table I. As expected, salts alone at these relatively low concentrations have no significant effect on the self-diffusion coefficient of water (McCall and Douglass, 1965). The organic solute, TMAO, exerts a remarkable reduction in the self-diffusion coefficient of water, $(d\mathcal{D}/dC_2)/\mathcal{D}_0$, being $\sim -0.30\ M^{-1}$, as shown in Fig. 2. Glycerol has a similar but less pronounced effect, with $(d\mathcal{D}/dC_2)/\mathcal{D}_0$ being $\sim -0.21\ M^{-1}$. Propionate, even at the

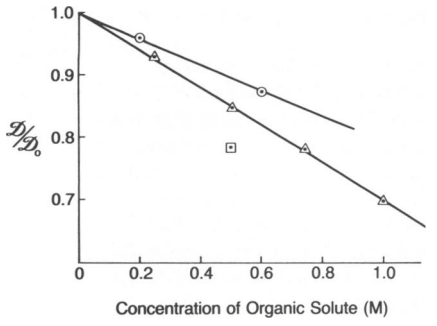

FIGURE 2 The relative self-diffusion coefficient of water, $\mathcal{D}/\mathcal{D}_o$, in solutions of the organic solutes glycerol and trimethylamine oxide (TMAO), as a function of solute concentration. Note that when 170 mM propionate is added to a 0.5 M TMAO solution there is a further lowering of $\mathcal{D}/\mathcal{D}_o$. ⊙, Glycerol; △, TMAO; ⊡, TMAO + 170 mM propionate (TM5-P/170).

relatively low concentration of 170 mM (+ 11.5 mM as buffer), also decreases the water self-diffusion coefficient.

$\mathcal{D}/\mathcal{D}_0$ of Protein Solutions

The measured self-diffusion coefficient of water in solutions of BSA (Table III) can be used to estimate the hydration of BSA using Eqs. 4–6. Assuming BSA to have the same shape, $\bar{\alpha}_p = 1.56$, and partial specific volume $\bar{V}_p = 0.746$, as ovalbumin (Wang, 1954), we obtain $H_{BSA} = 0.45$ g water/g protein. This number can be compared with that for ovalbumin, calculated from the data of Wang et al. (1954) and Eqs. 4–6; we obtain $H_{ovalbumin} = 0.60$ g water/g protein. Note that as Eq. 4 is valid for an infinitely dilute solution, the values of H obtained therefrom are lower limits. The theory of Schantz and Lauffer (1962) can be used to predict $H_{ovalbumin}$ for the case of more concentrated solutions. Again using Wang's data, we calculate from Eq. 5 of Schantz and Lauffer a value of $H_{ovalbumin} = 0.78$ g water/g protein.

TABLE III
T_2 AND $\mathcal{D}/\mathcal{D}_0$ FOR SOLUTIONS MIMICKING CELL SOLUTES

Solution	T_2*	$\mathcal{D}/\mathcal{D}_0$ observed‡	$\mathcal{D}/\mathcal{D}_0$ expected§
Salt-propionate			
S-1	2.047	0.942	—
S-2	2.029	0.905	—
Organic			
O-1	0.389	0.903	—
O-2	0.255	0.820	—
Protein			
P-1	0.950	0.959	—
P-2	0.596	0.899	—
P-3	0.304	0.829	—
Combinations			
S/P [(S-1) + (P-2)]	0.681	0.866	0.877
O/S [(O-1) + (S-1)]	0.382	0.863	0.856
O/P [(O-1) + (P-2)]	0.274	0.801	0.806
S/O/P [(S-1) + (O-1) + (P-2)]	0.276	0.775	0.769
Water (three-times distilled)	1.948	1.000	
Cell ultracentrifugate	0.6	0.802	
Cell ultracentrifugate corrected for lost high-molecular weight solutes	—	0.767	

*Errors in T_2 estimated at $< \pm 10\%$. ‡Errors in $\mathcal{D}/\mathcal{D}_0$ estimated at $< \pm 1\%$. §Values of $\mathcal{D}/\mathcal{D}_0$ expected were calculated using the relationship $\mathcal{D}/\mathcal{D}_0 = 1.0 + \Sigma_i [\partial(\mathcal{D}/\mathcal{D}_0/\partial c_i] c_i$.

$\mathcal{D}/\mathcal{D}_0$ of Sarcoplasmic Fluid

Our measurements of T_1 and T_2 of ultracentrifugate from fresh fibers indicate that T_1 (1.89 s) is not significantly different from a similarly oxygenated sample of water (1.87 s), but T_2 (0.6 s) is reduced approximately threefold. This is presumably due to contributions to the spin-spin relaxation from rapidly exchanging water molecules bound to dissolved macromolecules (Burnell et al., 1981). Our measurement of the self-diffusion coefficient of water in the cell ultracentrifugate gives the value 0.80 $\mathcal{D}_0$ (Table III).

$\mathcal{D}/\mathcal{D}_0$ for Various Combinations of Solutes

In Table III we report T_2 values and relative self-diffusion coefficients of various combinations of solutes found in barnacle muscle ultracentrifugate. We note that the effects of various categories of solutes on T_2 and $\mathcal{D}/\mathcal{D}_0$ are not parallel. Propionate-containing salt solutions have little effect on T_2, whereas both organic nitrogenous solutes and proteins increase the rate of the spin-spin relaxation in linear proportion to their concentrations (Fig. 3). This is probably due mainly to exchange of water protons with protons on free amino groups, whose proton resonance is broadened by the quadrupolar nitrogen nucleus (Pople et al., 1959). This is consistent with our finding that solutions up to 1 M of TMAO, which has no exchangeable protons, have T_2 values that are the same as for pure water. (Other relaxation processes are, of course, present in protein solutions, as discussed earlier [Burnell, et al., 1981].) If we assume that the contributions to T_2^{-1} from the various substituents of the complete ultracentrifugate (modeled by solution S/O/P, see Table III) are additive, then we predict a T_2 of 0.256 s, which compares well with the observed T_2 for S/O/P of 0.276 s.

It is clear from Table III that the small organic solutes found in the sarcoplasmic fluid significantly reduce the self-diffusion coefficient of water protons, as do TMAO, glycerol, and propionate reported in Table I. As shown in Figs. 2 and 4 *A*, the effects are approximately linear with solute concentration.

In Table III, the relative self-diffusion coefficients expected after combining groups of solutes are shown to agree well with the values actually observed. Thus, there are no unexpected interactions among the various solutes that affect the self-diffusion coefficient of water.

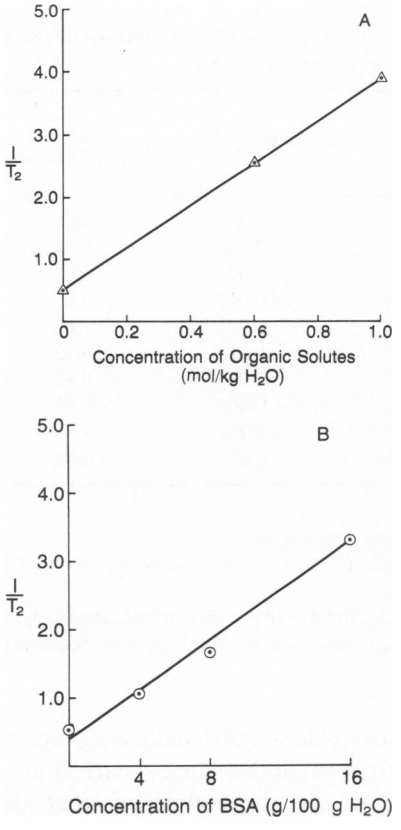

FIGURE 3 Spin-spin relaxation rates (T_2^{-1}) as a function of solute concentration. *A*, organic mixtures resembling barnacle muscle low molecular weight solutes. *B*, bovine serum albumin.

Our sarcoplasmic fluid sample containled only ~60% of the dissolved macromolecules that occur in the living cell; the rest sedimented out during the high speed ultracentrifugation necessary to separate the supernatant from the myofilaments (Clark and Hinke, 1981). By assuming that on a weight basis, these lost macromolecules contribute the same as BSA to the relative diffusion coefficient, we calculate a value of 0.767 for $\mathcal{D}/\mathcal{D}_o$ of the sarcoplasmic fluid. This compares well with the value of 0.775 for the artificial model solution, S/O/P, in Table III. Thus, our model mixture approximates the actual sarcoplasmic fluid very closely.

It is thus clear that when interpreting the reductions in both spin-spin relaxation rates and self-diffusion coefficients in living cells, it is necessary to consider contributions from soluble components in the cell which have usually been ignored. This is especially true for tissues from those euryhaline and marine species that tolerate osmotic pressures greater than ~350 mosmol, and hence possess high intracellular concentrations of amino acids and other small organic solutes (Clark and Hinke, 1981).

Calculation of $\mathcal{D}'$ for Various Fibers

Because small solutes within fresh or membrane-damaged fibers may have a marked effect on the self-diffusion coefficient of the solvent, this effect must be compensated before applying the diffusion equations to obtain estimates of macromolecular hydration. The values of the self-diffusion coefficient in the fibers are thus made relative not to the value for pure solvent, $\mathcal{D}_o$, but to the value for the solution within the fiber, $\mathcal{D}'$. For the membrane-damaged fibers, $\mathcal{D}'$ is taken as the value obtained on the appropriate equilibrating solution (Table I). As we showed earlier, the equilibrium concentrations of solutes in the fiber water and the bath are sufficiently similar to take them as equal for present purposes (Clark et al., 1981).

For the fresh fibers, $\mathcal{D}'/\mathcal{D}_o$ was taken as 0.854, the value that the supernatant would be expected to have if it contained no macromolecules. This value was obtained by subtracting out the contribution of the macromolecules (using the footnote equation of Table III and slope from Fig. 4 *B*) from the measured value of $\mathcal{D}/\mathcal{D}_o$ of the ultracentrifugate. Note that it compares very well with the observed value (0.863) for an artificial solution (O/S, Table III) containing salts and small organics in the same

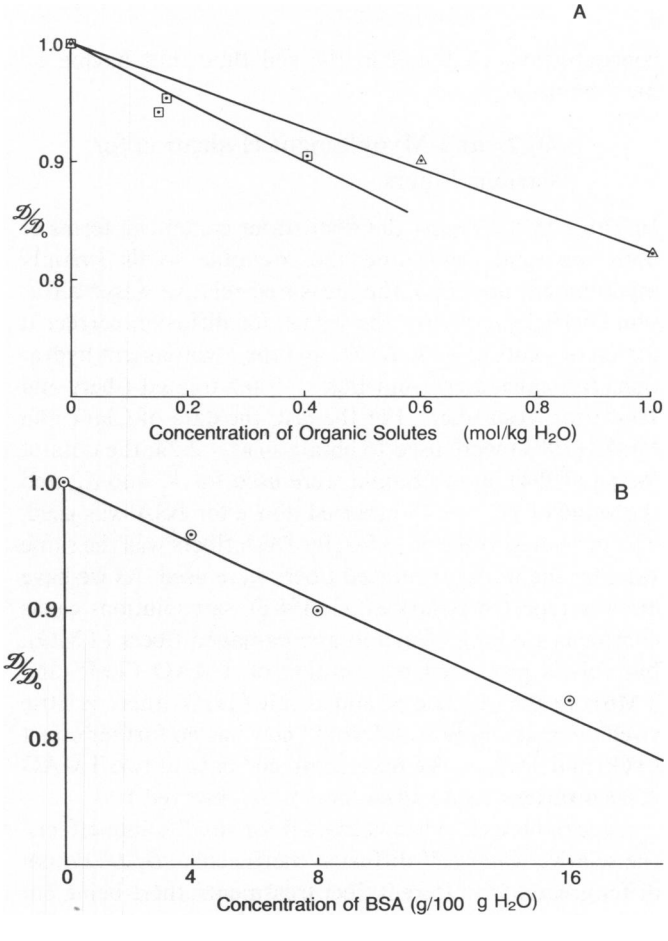

FIGURE 4 The relative self-diffusion coefficient of water, $\mathcal{D}/\mathcal{D}_o$, in artificial mixtures of solutes found in barnacle muscle as a function of concentration. *A*, salt-propionate solutions and small nitrogenous organic solutes. ⊡, Propionate-containing solutions (S–1 and S–2 and P–170). △, Solutions containing small organic molecules (O–1, O–2). *B*, bovine serum albumin.

TABLE IV

SELF-DIFFUSION PARAMETERS IN FRESH AND TREATED BARNACLE MUSCLE AND COMPARISON WITH MYOFILAMENT HYDRATION FROM RELAXATION EXPERIMENTS

Preparation (n)* (percent H_2O in fibers)	g H_2O/ g total solid	gH_2O/ g insoluble solid	$\mathcal{D}/\mathcal{D}_0$	$\mathcal{D}/\mathcal{D}'$	H_m‡ g bound H_2O/g macromolecule	H_r§
Fresh fibers (3)	3.01	5.87	0.551	0.646	0.65	0.07
(75.0 ± 0.2)	± 0.04	± 0.07	± 0.021	± 0.025	± 0.08	
TM5 and TM6 (2)	4.11	6.13	0.571	0.670	0.92	0.09
(80.4 ± 0.6)	± 0.16	± 0.15	± 0.012	± 0.011	± 0.07	
TO5 and TO6 (2)	5.11	7.69	0.609	0.721	1.00	—
(83.7 ± 0.1)	± 0.02	± 0.30	± 0.022	± 0.015	± 0.12	
TOO (2)	6.21	6.39	0.755	0.755	0.65	—
(86.1 ± 0.4)	± 0.20	± 0.22	± 0.022	± 0.002	± 0.03	
TMO (4)	10.1	12.6	0.802	0.768	1.46	0.07
(90.9 ± 0.8)	± 0.9	± 1.3	± 0.022	± 0.021	± 0.23	

All values are means ± standard errors.

*n is the number of samples, taken from different barnacles on different days, that make up each mean value.

‡H_m is g bound water per g myofilament protein in all samples, calculated from Eqs. 7–9 and 10–13. If equations more appropriate for concentrated solutions were used, larger values of H_m would be obtained.

§H_r is g bound water per g macromolecules from relaxation measurements (Burnell et al., 1981). In fresh fibers, this includes soluble macromolecules in the sarcoplasm; in other fibers it refers only to myofilament proteins. In the relaxation studies, it was assumed that the g water bound per g soluble and insoluble macromolecules was the same.

concentrations as found in the cell fluid, but having no macromolecules.

$\mathcal{D}/\mathcal{D}'$ and Myofilament Hydration for Various Fibers

In Table IV we report the fiber water content in terms of both the total solids and the insoluble solids (mainly myofilament proteins); the measured relative water diffusion coefficient, $\mathcal{D}/\mathcal{D}_o$; the values for diffusion corrected for small solute effects, $\mathcal{D}/\mathcal{D}'$; and the myofilament hydration, H_m, calculated from Eqs. 7–9 for treated fibers and 10–13 for fresh fibers. For the last, the data of Clark and Hinke (1981) were used to obtain $v/w = 2.24$; the data of Wang (1954) on ovalbumin were used for $\bar{V}_p$ and $\bar{\alpha}_p$; and the value of $H_p = 0.45$ reported above for BSA was used. The measured value of $\mathcal{D}/\mathcal{D}_o$ for fresh fibers was the same whether the whole or minced fibers were used. As we have already reported (Clark et al., 1981), salt solutions cause enormous swelling of membrane-damaged fibers (TMO), but this is prevented by addition of TMAO (TM5 and TM6). In the absence of added salt (TOO) there is little swelling, and addition of TMAO now has no further effect (TO5 and TO6). (We have combined data at two TMAO concentrations, as no differences were observed.)

As can be seen, when corrected for small solute effects, the relative water self-diffusion coefficients, $\mathcal{D}/\mathcal{D}'$, do not differ greatly for different fiber treatments, there being but a small tendency to increase as fiber water content increases. When the values for the myofilament hydration, H_m, in treated fibers are compared with that in fresh fibers, only those for salt-treated (TMO) fibers are significantly greater, being about twice the size. As previously reported, electronmicrographs of salt-treated fibers indicate that with increasing ionic strength there is a progressive disruption of myofilament architecture (Clark et al., 1981). It is not possible to say for certain whether the apparent increase in H_m in these fibers is due to a breakdown in myofilament geometry, so that our diffusion model no longer applies, or to a change in the interaction between the myofilament proteins and the surrounding water molecules leading to a true increase in H_m. Possibly, both factors are contributing. Further studies, of the sort reported in Table III, are needed at higher salt concentrations to see whether denaturing salt concentrations produce nonadditive effects of $\mathcal{D}/\mathcal{D}'$ when added to model protein solutions, presumably signifying changes in H_m with protein unfolding.

The Nature of Water in Fresh Fibers

Before considering our results on macromolecular hydration, it is important to keep in mind two important factors: (*a*) the model by which the data are interpreted and (*b*) the quantity that is actually being measured. With regard to the first, as already noted, we hold Wang's diffusion model incorrect, and have adopted Lauffer (1961) and Rorschach and Hazlewood's modification.[2] Using the model that Wang developed, he and his colleagues (Wang et al., 1954) obtained a value of 0.18 g water bound/g ovalbumin; using their data and our modified diffusion model, we obtain a value of 0.60 g/g. It is thus clear that the choice of model by which the data are interpreted can have a substantial effect on the calculated hydration.

The second factor is the quantity actually being measured, which in turn depends upon the nature of the experiment. This question has been addressed by Berendsen (1975) who pointed out the confusion that arises when "hydration" or "bound water" is measured by tech-

niques that sample quite different properties of water. He notes that there are three major aspects of hydration: (*a*) thermodynamic aspects, relating to enthalpies and entropies of molecular interactions; (*b*) dynamic aspects, dealing with molecular motions; and (*c*) structural aspects, relating to the geometric arrangement of solvent molecules. We shall confine our comments to the question of molecular motions, noting that this is not necessarily related to solvent, osmotic, or freezing properties of the system, all of which may more properly be considered as thermodynamic aspects.

In our fibers, when we compare the amount of water bound to macromolecules that is measured by diffusion, H_m, with that measured by relaxation, H_r, (see last column in Table IV, data from Burnell et al., 1981), there is approximately a one-order-of-magnitude discrepancy. It is therefore necessary to define more precisely the kinds of motions that each method is capable of detecting.

In our relaxation studies (Burnell et al., 1981), the primary contributions to relaxation were interpreted to come from a small number of water molecules bound to macromolecules, and tumbling anisotropically with a correlation time $\tau_b \simeq 1.3 \times 10^{-8}$ s. The bound water molecules exchange with free water with a time constant of $\tau_{ex} \simeq 8 \times 10^{-6}$ s. These motions, which are slow compared to the rotational correlation time for pure water at the same temperature ($\tau_c \simeq 5 \times 10^{-12}$ s), efficiently relaxed all signals, even at the highest frequencies employed ($\sim 10^9$ s^{-1}). Therefore, modest decreases, by a factor of two or three, in rotational or diffusional correlation times of isotropically moving water would not be observed in our relaxation experiments. In marked contrast, however, our diffusion measurements would be highly sensitive to changes in such high frequency motions. It thus appears that we are now sampling the effect of nonaqueous cell components on a much larger fraction of cell water.

We now raise the question, however, whether it is reasonable to speak in terms of "macromolecular hydration" when considering the reduced self-diffusion of water in muscle cells. The term hydration suggests a discrete or bounded fraction of water molecules, the diffusional motions of which are reduced by at least five or six orders of magnitude. If this picture were adopted, our present diffusion measurements and the model for interpreting them as "macromolecular hydration" would create a nearly stationary layer several molecular diameters in thickness. This is in direct contradiction, however, to our relaxation measurements, which indicate only a monomolecular layer of anisotropically tumbling molecules with severely restricted motion (Burnell et al., 1979, 1981).[3] We thus conclude that it is not correct to interpret our diffusion data in terms of a static shell of bound water molecules.

An alternate model is one in which there is a gradient of diffusional motions that are virtually zero at the surface of stationary macromolecules and become greater at increasing distances from the surface. The number of affected water molecules would depend upon the steepness of the gradient. It is conceivable that all of the water molecules in our muscle fibers could be experiencing some degree of reduced diffusional motion. NMR diffusion measurements alone cannot identify the exact fraction of molecules involved, but information could in principle be obtained from T_1 measurements at Larmor frequencies near the characteristic frequency for this motion. Perhaps one day the techniques for such measurements will become available.

We note that extensive alteration of the structure of cell water which our results imply has also been proposed by Hazlewood and colleagues from studies on the self-diffusion of water in rat muscle (see Hazlewood, 1979, for review) and has been predicted by Morel and Gingold (1979) in their analysis of the factors that stabilize the myofilament lattice. The latter workers argue that the conventionally proposed balance between van der Waals–London dispersion forces and electrostatic repulsion is insufficient, and they invoke an active role for the intervening solvent molecules. Previously, such a role has generally been sought among the low-frequency motions reflected in reduced rotational freedom and measured by rapid-transverse relaxation processes. Now, however, it appears that proteins (and also organic solutes) may strongly affect the translational motions of water molecules (and hence the solvent structure) over long distances without greatly altering their rotational freedom.

Such a long-range effect of macromolecules on the surrounding solvent becomes even more plausible when we consider that, weight for weight, myofilament proteins, dissolved albumins, and small organic solutes have approximately the same effect on the relative self-diffusion coefficient of water. It is possible that in all three systems, similar changes in water structure are wrought by the presence of nonpolar groups. That small, nonelectrolyte solutes can affect the rotational and diffusional motions of water independently has been demonstrated by Goldammer and Hertz (1970). For various solutes of this type these workers showed that semi-permanent hydration spheres do not exist; instead, the solutes appear to increase the structure of the solvent over long distances. An explanation has been offered by Zeidler (1973), who reviewed the effects of nonpolar alkyl groups on the NMR-determined parameters of water motion. He proposed that the surrounding solvent molecules do not interact directly with these solutes, but are constrained to form stronger or more numerous hydrogen bonds among themselves. This model is a restatement of the so-called hydrophobic hydration effect described by Tanford (1973). If this is indeed the primary cause of the extensive influence on solvent self-diffusion of a wide variety of organic solutes and macromolecules, then altered conditions of temperature and pressure should result in predictable changes in solvent

[3]Note that the incorrect Wang equation was used in the 1979 study.

motions (Ben-Naim, 1980). We are undertaking a study of model systems to investigate this possibility.

APPENDIX

Justification of Eq. 4

Eq. 4 differs from the obstruction equation proposed by Wang (1954) by the factor $(1 - \phi)$ in the denominator. This factor accounts for the volume fraction of material that is not free to diffuse; it should be included for analysis of diffusion measurements using any technique, including both NMR field gradient and radio-isotope tracer methods. It is not restricted to the NMR method. The error in Wang's theory is that the concentration of diffusable material is used in an inconsistent manner. The Wang model for obstruction is based on a calculation of the diffusional flow, q, along the long axis, x, of a cylinder connecting two baths that contain different concentrations of labeled water. Examples of labels are radio-isotopes and, for NMR, the magnetizations of nuclear spins. Steady-state conditions are assumed such that the concentration, c, of the label in the cylinder at distances far from the obstruction can be written as

$$c = \bar{c} + c'x \qquad \text{(Wang 5)}$$

where $\bar{c}$ is the average concentration of label at $x = 0$, and c' is the concentration difference of the label in the two baths divided by the length of the cylinder. Fick's first law then gives the average diffusional flux of labeled molecules in the x direction as

$$q = -\frac{\mathcal{D}_0}{V}\int_V \frac{\partial c}{\partial x}\,dV. \qquad \text{(Wang 8)}$$

In this equation, q has the units moles of label per cross-sectional area per unit time, and the integration is carried out over the entire volume, V, of the cylinder. The cross-sectional area must include the average area occupied by the obstructions.[2] In this equation, c must be the concentration of label per volume of diffusing material, i.e., the volume must exclude the non- (or slowly-) diffusing macromolecules. In the presence of infinitely dilute obstructions, Wang's Eq. 8 becomes

$$q = -\mathcal{D}_0 c'(1 - \bar{\alpha}\phi) \qquad \text{(Wang 12)}$$

where $\bar{\alpha}$ and ϕ have the meanings assigned in the body of this paper. Wang (1954) then defines an effective self-diffusion coefficient, $\mathcal{D}$, of the free water molecules in the protein solution by

$$q = -\mathcal{D}\gamma' \qquad \text{(Wang 13)}$$

in which we have replaced his c' by the value γ'. In order for Eqs. (Wang 12) and (Wang 13) to be equivalent, i.e., for q in (Wang 13) to have the units of moles of label per cross-sectional area per unit time, γ' must be the average concentration gradient of the label for the total volume, including protein. Hence, we must equate

$$\gamma' = (1 - \phi)c' \qquad \text{(A1)}$$

and then (Wang 13) becomes

$$q = -\mathcal{D}(1 - \phi)c'. \qquad \text{(A2)}$$

The presence of obstructions leads to an effective diffusion constant of

$$\mathcal{D} = \mathcal{D}_0\frac{(1 - \bar{\alpha}\phi)}{(1 - \phi)}. \qquad \text{(A3)}$$

It is now straightforward to show that this equation along with the effect of hydration leads to Eq. 4, where ϕ must then be the hydrated protein volume. Note that this equation holds true whether or not the obstruction also occurs in the baths, because the measurement in either case is of q, the flux within a cylindrical volume. Further demonstration of the correctness of this interpretation can be found in the measurement of diffusion parallel to infinitely long rods. For this case, $\alpha_\parallel = 1$ (Wang, 1954). Obviously, there are no obstructions to diffusion and the diffusion constant is not affected in this case. The flow, q, however, is reduced by the excluded volume effect. With the corrected Eq. 4, $\mathcal{D}$ and $\mathcal{D}_0$ are equal, as expected.

We would like to thank Mr. Laurie Nicol and Mr. Alan Kilpatrick for technical assistance, and Mr. Aristotle Azad for protein analysis on the fiber ultracentrifugate. We appreciate the opportunity to study an unpublished manuscript of Professors H. E. Rorschach and C. F. Hazlewood regarding the model for interpretation of diffusion measurements in muscle fibers, and value the exchange of comments. Special thanks are due to Professor Myer Bloom of the Physics Department not only for logistical support (e.g., ice cream cones) but also for many invaluable discussions, and to Dr. Paul Callaghan for critically reading our theoretical arguments. We also acknowledge the useful comments of the referees.

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