THE STATE OF WATER IN MUSCLE TISSUE AS DETERMINED BY PROTON NUCLEAR MAGNETIC RESONANCE

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ABSTRACT The nuclear magnetic resonance (NMR) of water protons in live and glycerinated muscle, suspensions of glycerinated myofibrils, and solutions of several muscle proteins has been studied. T_1 and T_2, measured on partially hydrated proteins by pulsed spin-echo techniques, decreased as the ratio of water to protein decreased, showing that the water which is tightly bound by the protein has short relaxation times. In live muscle fibers the pulse techniques showed that, after either a 180 or a 90° pulse, the relaxation of the magnetization is described by a single exponential. This is direct evidence that a fast exchange of protons occurs among the phases of the intracellular water. The data can be fitted with a model in which the bulk of the muscle water is in a phase which has properties similar to those of a dilute salt solution, while less than 4–5% of the total water is bound to the protein surface and has short relaxation times. Measurements of T_1 and T_2 in protein solutions showed that no change in the proton relaxation times occurred when heavy meromyosin was bound to actin, when myofibrils were contracted with adenosine triphosphate (ATP), or when globular actin was polymerized.

INTRODUCTION

Although water comprises 70–80% of all living systems, considerable controversy still exists about the structure of water inside living cells. One hypothesis is that the bulk of the intracellular water has properties, viscosity, degree of hydrogen bonding, etc., similar to those of a dilute salt solution (for the purpose of this paper such water will be termed "free water") and that a minor fraction of the water has altered properties because of its strong interaction with the proteins (1). This minor fraction, sometimes termed the water of hydration of the proteins, is estimated from in vitro studies on proteins to be of the order of magnitude of 20% of the protein weight. Thus for muscle fibers, which are about 20% protein, this minor phase would comprise only about 4–5% of the total water. Evidence that little additional structure has been imposed on the bulk of the intracellular water comes from studies which

show that the self-diffusion of intracellular water in several plant and animal tissues, when corrected for the presence of protein microbarriers, is similar to that of water in a normal phase (2, 3); moreover, it has been shown that almost the entire intracellular medium is accessible to diffusion of dimethyl sulfoxide (DMSO) (4). A second hypothesis, however, suggests that the bulk of the intracellular water is in a more highly structured phase, or phases, than water in a dilute salt solution. This hypothesis is based on vapor pressure studies which have been interpreted to mean that 95% of muscle water exists in a structured form, the remaining 5% comprising a qualitatively still more tightly bound phase (5) and on NMR studies which have shown that water protons have wider line widths in muscle (6), and that water deuterons have shorter relaxation times in muscle and brain than in free water (7).

The question of the structure of intracellular water is of fundamental importance to many fields of molecular biology, but is of particular importance to the problem of muscle structure and function. Elliott et al. (8) have recently suggested that long-range electrostatic forces play a role in maintaining the structure of the filament lattice and have implied that the intrafilament water is structured and osmotically inactive. Other workers (9) have presented models in which electrostatic forces generate the tension which contracts muscle. Changes in the charge of proteins could also cause changes in protein hydration which, if detected, could provide clues to the events which cause contraction.

The present study investigates the NMR spectral line widths, chemical shifts, and relaxation times of water protons in live and glycerinated muscle fibers and in solutions of various muscle proteins. We conclude that the bulk of the intracellular water has an NMR spectrum similar to free water, that a small fraction (less than 4–5% of the total water content of the fibers) has short relaxation times, and that a fast exchange between the two fractions can explain the observed spectra for intracellular muscle water.

METHODS

The chemical shifts and line widths were measured at 100 MHz on a Varian HA-100 NMR spectrometer (sample temperature 33 ± 2°C), at 60 MHz on a Varian T-60 (35 ± 2°C), and at 30 MHz on a Varian EM-300 (28 ± 2°C) (Varian, Palo Alto, Calif.). A capillary of tetramethylsilane (TMS) was used as a reference signal. The same capillary was used for all samples. The chemical shift was determined by interpolation between markers which were calibrated in terms of the difference in frequency between the sweep and manual oscillators. T_1 (the longitudinal or spin-lattice relaxation time) was measured at 100 MHz by the adiabatic rapid passage technique (10). Each T_1 represents the average of at least five measurements.

Measurements of T_1 and T_2 (the transverse or spin-spin relaxation time) at 51.6 MHz were made on a Magnion ELH-15 pulsed spectrometer (Magnion Div., Ventron Instruments Corp., Burlington, Mass.) equipped with a Varian 9-inch, high-resolution magnet with flux stabilizer. T_2 measurements were made using the Meiboom-Gill modification (11) of the Carr-Purcell spin-echo method, with a 5 msec pulse spacing unless otherwise specified (12). The T_1 values were obtained, using the 180-90° pulse sequence, from the slope of a semilog plot of the recovery of the magnetization (12). The sample temperature was 25 ± 2°C.

Myosin A was extracted with a modified Szent-Györgyi method (13), and heavy meromyosin (HMM) was subsequently prepared by the method of Mihalyi and Szent-Györgyi (14). Actin was extracted by the acetone powder method (15). Rabbit psoas fibers were glycerinated at −20°C for 1–4 months in a 50% glycerol-water solution with 0.05 M KCl and 0.01 M histidine, pH 7.0. These fibers were homogenized in a Sorvall Omni-mixer (Ivan Sorvall, Inc., Norwalk, Conn.) at full speed for 60 sec, washed three times in 0.08 M KCl, 0.01 M histidine, pH 7.0, and strained through two layers of cheesecloth. The resulting suspension consisted of myofibrils approximately 5 μ in diameter and varying in length from 10–100 μ. Rabbit psoas fibers were dissected at approximately rest length and kept on ice until measured; all measurements on living fibers were made during the relaxed state. Protein concentrations were determined by the biuret method (16).

RESULTS AND DISCUSSION

The properties of water in biological systems have been extensively studied by the use of NMR. The parameters which are usually employed are the width and integrated intensity of the water peak in the steady-state proton spectrum, the chemical shift, and the relaxation times T_1 and T_2. The steady-state proton NMR spectrum of free water at 100 MHz consists of a single line with a full width at half-height of 2.5 Hz. The relaxation times are both equal to 3.0 sec. Previous workers have shown that water protons or deuterons in protein solutions (17, 18), in nucleic acid solutions (19), or inside plant and animal tissues (7, 20) have broader steady-state spectra and shorter relaxation times than in free water. These workers have excluded paramagnetic impurities as being a source of the changes in spectra.

Daszkiewicz et al. (21) were the first to study the concentration dependence of relaxation times in protein solutions. They showed that for protein concentrations less than 100 mg/ml the observed relaxation times could be fitted to an equation of the form:

$$T_1^{-1} = T_1(w)^{-1} + k_1 c, \tag{1}$$

where T_1 is the observed relaxation time, $T_1(w)$ is the relaxation time of water, k_1 is an experimental constant, and c is the concentration of the protein in grams protein per gram H_2O. A similar equation holds for T_2. By assuming that a fast exchange occurs between some water that is rigidly bound to the protein surface and free water of the solution, they were able to make a rough calculation of the constant k_1.

T_1's were measured by adiabatic rapid passage, and steady-state spectra of suspensions of myofibrils, of live and glycerinated fibers, and of protein solutions were taken at 100 MHz. For all of these samples a simple exponential curve was observed for the return of the magnetization to its equilibrium value, and a single broad peak was observed for the steady-state spectrum. This is the behavior which is to be expected if a fast exchange occurs between protons inside the fibers and those in the surrounding medium. If such an exchange did not take place within the relaxation time of the protons inside the fibers, two relaxation times would be observed for T_1 and two peaks would be observed in the steady-state spectrum. For both the fiber

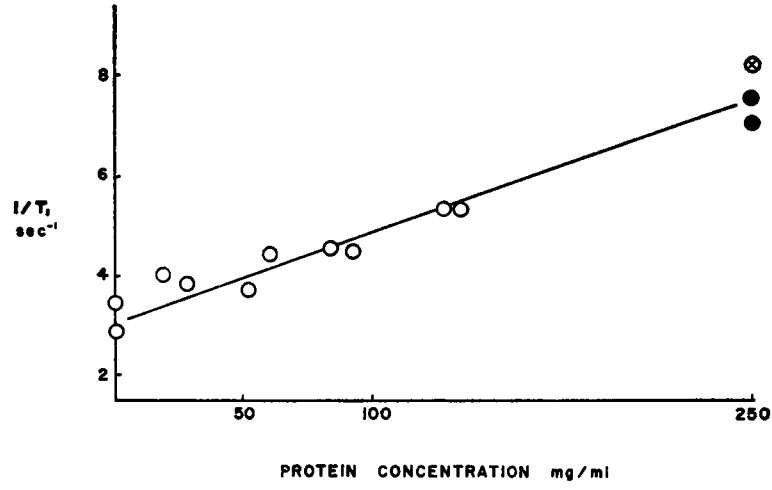

FIGURE 1 The inverse of the spin-lattice relaxation time, determined by the adiabatic rapid passage at 100 MHz, of water protons in suspensions of glycerinated myofibrils and in live and glycerinated fibers as a function of the protein concentration. Myofibrils, ○; live fibers, ⊗; and glycerinated fibers, ●.

TABLE I

VALUES OF k_1, CALCULATED FROM EQUATION 1 USING THE OBSERVED T_1^{-1}'S, FOR THE VARIOUS SAMPLES STUDIED

Sample	k_1
Myosin A, 0.6 M KCl	3.4 ± 0.2
Myosin A, 0.08 M KCl	2.5 ± 0.2
G-actin	1.6 ± 0.2
F-actin	1.6 ± 0.2
Actomyosin, 0.08 M KCl	2.3 ± 0.2
Actomyosin (calculated)	2.1 ± 0.2
Muscle fiber	1.8 ± 0.2

and the protein samples T_1 followed a dependence on the protein concentration which could be described by equation 1 (Fig. 1). Thus the proton longitudinal relaxation rates $1/T_1$ are directly proportional to the protein concentration. The constant k_1 (equation 1) defines the strength of whatever protein-water interaction is responsible for the increase in the water proton relaxation rate; the greater k_1, the stronger is this interaction. Since the T_1's for either live or glycerinated fibers fall on the same line as those for the myofibrils, we can conclude that the relaxation mechanisms which operate inside the fibers give rise to relaxation rates which are proportional to the protein concentration. The k_1's found for the fibers as well as for several solutions of muscle proteins are summarized in Table I. The k_1 for the fibers falls in the same range as the k_1's for the protein solutions. Thus we can fur-

ther conclude that the relaxation mechanisms operating inside the live muscle fibers are similar to those which operate in the protein solutions.

The study of intracellular water structure has been hampered by the fact that many direct physical measurements such as viscosity cannot be performed inside cells, and most of our knowledge on protein-solvent interactions comes from studies done on proteins in vitro. No unique definition exists to delineate exactly how much water interacts with a protein, and the amount found depends on the method employed. Kuntz et al. (1) have tabulated results for several proteins: NMR spectra show that 0.31–0.45 g H_2O/g protein does not freeze at −35°C, 0.45–1.0 g H_2O/g protein is the maximum amount of water carried by a protein during brownian rotations, and 0.25–0.32 g H_2O/g protein has a lower vapor pressure than free water. Wang (22, 23) has estimated that 0.18 g H_2O/g protein is not available for the self-diffusion of water in ovalbumin solutions. Cheung et al. (24) using a fluorescent dye to measure the rotational relaxation of G-actin, put an upper limit of about 0.5 g H_2O/g protein on the amount of water carried by the protein through its brownian rotations. In addition, the viscosity of a G-actin solution is that which is expected by assuming that spherical actin molecules having the correct molecular weight move in a medium having the viscosity of free water. All of the above results indicate that in a protein solution the bulk of water is free and from 0.2 to 0.5 g H_2O/g protein interacts with the protein and has properties which are different from free water. As an operational definition we will define this water which interacts with the protein to be the hydration sphere of the protein.

From Table I we see that the k_1 for fibers is little different from those for the proteins, and, in particular, that it is close to that of G-actin for which we have just cited some physical measurements on the degree of hydration. From the similarity of the k_1's we have concluded that the interactions between proteins and water is little different inside a muscle fiber than in vitro and thus that the water-protein interactions already known from in vitro experiments can account for the NMR spectra seen inside the fibers. Since the viscosity measurements show that there is no water structure extending beyond the hydration sphere of the protein in a G-actin solution, and since the dependence of the water proton relaxation rates on the protein concentration is similar in G-actin solutions and in muscle fibers, there is no need to invoke new concepts involving extensive water structure to explain the broadening of the steady-state spectra or the shortening of the spin-lattice relaxation time of protons inside muscle tissues.

The chemical shifts of the steady-state proton spectra can also give information on water structure. The greater the electron density surrounding a nucleus the more the nucleus is shielded from an external field, and the higher the field strength at which the nuclear resonance will occur. This effect has been used to study the properties of hydrogen bonds in various solutions. A proton involved in a hydrogen bond assumes a position somewhere midway between the donor and acceptor molecules in a region of lower electron density, thus causing a large downfield shift in its

resonance. For instance, an upfield shift of 4.6 ppm occurs when water goes from the liquid to vapor phase (25) and an upfield shift of approximately 5.0 ppm accompanies the breaking of hydrogen bonds by various ions (26).

The chemical shift of glycerinated fibers was measured by using an external standard (TMS) and also by addition of an internal standard (DMSO, less than 1 mM) to the fibers themselves. Shifts measured by the external standard had to be corrected for the volume susceptibility χ_v of the protein; χ_v was taken as -0.54×10^{-6} emu/g, a value measured for globin (27). The shift observed for the external reference was 4.4 Hz upfield from free water which agreed well with the upfield shift of 3.6 Hz observed for the internal reference.

If a large change occurs in the bulk of the muscle water, we would expect to see a large shift in peak position because of changes in the net number of hydrogen bonds. For instance when water is heated from 0 to 100°C there is a 0.95 ppm shift upfield (25); however, the shift that we see, 0.03–0.04 ppm, is very small compared to the shift which occurs on hydrogen bond formation and is also small compared to the shift seen on a 100°C increase in water temperature, indicating that there is little difference between the hydrogen bonding of free water and water inside muscle fibers.

The line widths of the steady-state spectra of the myofibril suspensions were linearly dependent on the protein concentration with a proportionality which was similar to that of live fibers or of the protein solutions. The basis for studying line widths in NMR spectra is the relationship between molecular mobility and line width. In steady-state absorption NMR the apparent T_2 is given by T_2^* , $= 1/\pi\Delta\nu_{1/2}$ where $\Delta\nu_{1/2}$ is the line width at half-height. This T_2^* has been related by some workers (6) to the correlation time which reflects molecular mobility; however, other line-broadening mechanisms, such as local field inhomogeneities, can give erroneous results so that measured line widths do not reflect the state of molecular mobility.

The Carr-Purcell sequence with the Meiboom-Gill modification can be used to measure T_2 in the presence of diffusion effects yielding a value of T_2 which is independent of local magnetic inhomogeneities and translational diffusion effects. Comparison of the value of T_2^* calculated from our line width experiments with T_2 measured by the Carr-Purcell method shows that, for live muscle, T_2^* equals 0.1 T_2 . Thus, the line width data imply little mobility in the intracellular water whereas the spin-echo data imply that the water is fairly mobile.

If the broad line width observed for the fibers is caused by local magnetic inhomogeneities, it should have a field dependence similar to that observed by other workers (28, 29). We found the line width at three frequencies, 30, 60, and 100 MHz, to be 5.5, 7.1, and 9.5 Hz respectively, displaying the expected linear field dependence.

The data taken at 100 MHz, T_1 and measurements of the chemical shift, indicate that the NMR spectra of muscle can be explained by assuming that there is a small hydration sphere having a short relaxation time and that a fast exchange occurs be-

tween this sphere and the rest of the water. Direct measurements with a faster time resolution can be made using pulse methods.

To investigate the properties of the various water phases inside the fiber, the relaxation times of partially hydrated samples were measured. A suspension of glycerinated fibrils was dehydrated by evaporation of the water into a vacuum. It has been shown that as the water content decreases the vapor pressure also decreases and that at low vapor pressures the remaining water is that which is tightly bound to the protein. Aliquots of a glycerinated fibril suspension were lyophilized after the initial ionic strength was set at 1 mM to avoid high salt concentration after removal of most of the water. After 5–6 hr of dehydration the vapor pressure had reached about 30 μ and the weight had become constant; this was taken as the "dry weight" of the sample. The water content of a given sample was determined on a similar aliquot. The steady-state spectrum of these samples at 100 MHz showed a broad peak with a half-width of 400–500 Hz and an amplitude which was approximately equal to the water content estimated from the weight of the sample. The relaxation times for these samples are shown in Table II. As the water content diminishes the relaxation times decrease, indicating that the more tightly the water is bound the shorter is T_1 or T_2.

A theory which describes the expected behavior of the relaxation times in a multiphase system has been developed by Zimmerman and Brittin (30). They describe two limiting cases for a two-phase system. In one case, there is a slow exchange between the phases defined as

$$D_i \ll 1/T(i),$$

where D_i is the reciprocal lifetime of the nucleus and $T(i)$ is the relaxation time, either spin-spin or spin-lattice, in the ith phase; and in the other case, there is a fast exchange between the phases, defined as

$$D_i \gg 1/T(i).$$

TABLE II

WATER PROTON RELAXATION TIMES T_1 AND T_2 MEASURED BY PULSE TECHNIQUES AT 51.6 MHz ON PARTIALLY HYDRATED MUSCLE FIBERS*

Amount of water	T_1	T_2
g H_2O/g protein	*msec*	*msec*
~0.05	25→	<5
0.20	45–78	11–18
0.50	125	24
1.0	200	42
Glycerinated fiber	560	66

* The data for a whole glycerinated fiber are shown for comparison.

In the presence of slow exchange, the nonequilibrium value of the transverse or longitudinal magnetization δM decays as the sum of two exponentials:

$$\delta M = M_0[P(a)e^{-t/T(a)} + P(b)e^{-t/T(b)}], \tag{2}$$

where $P(a)$ and $P(b)$ are the probabilities that the nucleus is found in phase a or b having relaxation times $T(a)$ and $T(b)$ respectively. In the presence of fast exchange a single exponential decay is observed:

$$\delta M \sim M_0 e^{-t/T(\text{avg})}, \tag{3}$$

with a relaxation time, T(avg) given by:

$$1/T(\text{avg}) = [P(a)/T(a)] + [P(b)/T(b)]. \tag{4}$$

The decay of both the transverse and the longitudinal magnetization for protons in a glycerinated fiber is shown in Fig. 2. Both T_1 and T_2 follow a single exponential decay over the time observed, obeying equation 3 rather than equation 2. Since the data of Table II show that some muscle water has a faster relaxation than that observed in the whole fiber, the simple exponential decay of Fig. 2 can only result if a fast exchange occurs between the various phases.

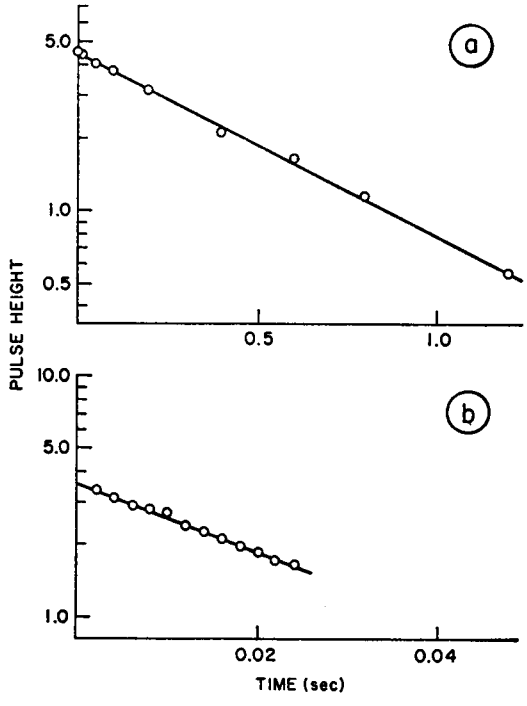

FIGURE 2 (*a*) The height of the signal seen after a 180-90° pulse pair as a function of time for water protons in a glycerinated muscle fiber. (*b*) The height of the echo seen after the 180° pulse of a sequence of 180° pulses after an initial 90° pulse.

Several observers have interpreted a loss of NMR signal intensity to indicate the presence of a tightly bound phase of water having a very fast relaxation time. The signal from this phase would supposedly decay before the first measurement of magnetization was made in Fig. 2 *a* 10 msec after the initial pulse. The decay of the longitudinal magnetization in Fig. 2 *a* is given by

$$M_z = M_0(1 - 2e^{-t/T_1}), \tag{5}$$

which extrapolates to $M_z = -M_0$ at $t = 0$ (10).

The signal height of Fig. 2 *a* extrapolated to zero time, is equal to that expected from the theory, i.e. is equal to $-M_0$, where M_0 is the equilibrium magnetization along the static magnetic field measured in the absence of a 180° pulse. Thus, there does not appear to be a missing water phase in the data of Fig. 2; however, if the protons in a water phase have a transverse relaxation time of less than 200 μsec, the length of the 90° pulse plus the dead time of the "receiver," the presence of this phase would not be detected because the proton resonance from this phase would not contribute to the magnetization measured by the pulse methods. The steady-state spectra of the partially hydrated samples have a full width of approximately 500 Hz, which implies a T_2 and therefore a T_1 of greater than 0.6 msec. Since this signal is proportional to the amount of water in the sample, we expect that it represents most of the hydration water. Thus, if some amount of water has been missed in Fig. 2 because of relaxation time less than 200 μsec, it represents only a small amount of the hydrated samples; assuming that we have missed 25% of the water in the hydrated samples, this lost phase will amount to only about 1% of the total muscle water.

Having shown that a fast exchange occurs among the different phases of muscle water, the theory of Zimmerman and Brittin can be used to see if the relaxation rates found in the hydration sphere are sufficient to explain the relaxation rates observed for the entire muscle. We can rewrite equation 4 as:

$$1/T\ (\text{avg}) = \frac{1 - C - Cx}{(1 - C)T_1(w)} + \frac{Cx}{(1 - C)T_1(b)}, \tag{6}$$

where C is the fraction of protein in the solution by weight, x is the amount of water in the hydration sphere in grams H_2O per gram protein, $T_1(w)$ is the proton relaxation in free water, $T_1(b)$ is the proton relaxation in the solvation layer, and a similar equation holds for T_2. Note that if $C \ll 1$, equation 6 becomes

$$1/T\ (\text{avg}) = \frac{1}{T_1(w)} + \frac{x}{T_1(b)}\,C, \tag{7}$$

which is equation 1 with $k_1 = x/T_1(b)$.

We can now use the measured value for $T(b)$ and equation 6 to calculate the expected relaxation times for fibers in the case of fast exchange. Assuming that the hy-

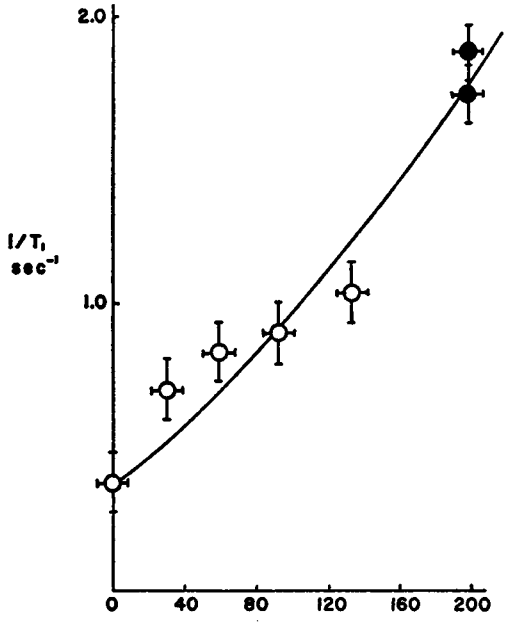

FIGURE 3

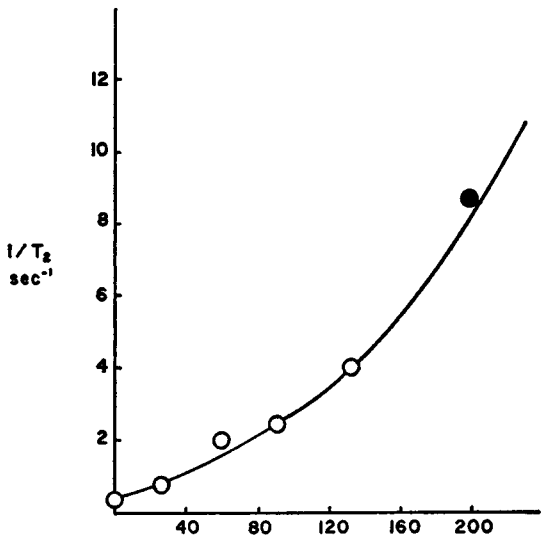

FIGURE 4

FIGURE 3 $1/T_1$ determined by pulse-echo techniques at 51.6 MHz for water protons in myofibril suspensions, ○, and in glycerinated fibers, ●, as a function of the protein concentration. The solid lines are the calculated values assuming fast exchange between a phase of water which has a relaxation time of free water and comprises 96% of the muscle water and a phase of water, bound to the proteins, which comprises 4% of the muscle water and has a relaxation time measured for the hydrated protein sample (Table II) with 0.2 g H_2O/g protein.

FIGURE 4 $1/T_2$ for water protons in suspensions of glycerinated myofibrils, ○, and in glycerinated muscle fibers, ●, as a function of protein concentration. The solid line is the theoretical curve calculated as in Fig. 3.

dration sphere amounts to 0.2 g H_2O/g protein and using the $T_1(b)$'s and $T_2(b)$'s measured for the sample having this amount of water, the curves shown in Figs. 3 and 4 were calculated. The excellent agreement between the data and the curves shows that the relaxation rates seen in glycerinated or live fibers or in fibril suspensions is explained by the relaxation rates found in this hydration sphere along with the presence of fast exchange.

Since the relaxation times continue to decrease as x decreases, the data of Table II do not clearly define the limits of the hydration sphere and the relaxation may be occurring in a sublayer within the hydration sphere. The agreement between theory and data in Figs. 3 and 4, however, shows that the water outside of this hydration sphere has a relaxation time similar to that of free water.

The results have implications for theories of muscle structure and function. Our re-

sults indicate that the bulk of the water inside a muscle fiber is free, and that the water having fast relaxation rates which imply less mobility is confined to the water in the hydration sphere of the proteins, comprising only about 4% of the total water content. Thus, any theory which implies that a large fraction of the water inside the muscle fiber be structured (for example, reference 8) is untenable.

The role which electrostatic interactions are thought to play in some of the interactions of muscle proteins has led several investigators to study the hydration of these proteins under varying conditions. Most often hydration has been inferred from volume measurements; an increase in volume for a given system is believed to be associated with a decrease in hydration since a hydrated or electrostricted water molecule occupies less volume than one in free water. Volume changes have been measured via dilatometry or have been inferred from the pressure dependence of the free energy. If an increase in pressure drives a reaction forward, the products should have a smaller volume and therefore a greater hydration than the reactants.

The most striking change seen in our parameters which can be attributed to a change in protein hydration is the decrease seen in the T_1 when the ionic strength of a myosin A solution is increased (see Table I). Myosin in 0.06 M KCl is aggregated into "thick" filaments which can be dissolved by raising the ionic strength to 0.6 M KCl. If the aggregation of myosin at low ionic strength buries charged groups within the thick filament, we would expect that dissolving the filament would result in an increase in hydration. Thus, the shorter T_1 seen at high ionic strength which indicates greater hydration seems reasonable. Unfortunately, this system has not been studied by other methods.

The force of muscle contraction is probably produced by the interaction of two proteins, actin and myosin. Two studies have investigated the volume changes of the proteins during their interaction; Rainford et al. found that high pressure inhibited the ATPase and superprecipitation of the two proteins (31), and Ikkai and Ooi (32) further showed that pressure favored their dissociation. Both results can be interpreted as an indication that the association of the two proteins results in a decreased hydration. Measurements of T_1 at 100 MHz (Table I) show that within experimental error no change occurs in the proton relaxation rate when myosin A binds to F-actin. This experiment was repeated with greater sensitivity by using HMM, the portion of myosin which contains the actin-binding site. T_1 and T_2 were measured by pulse methods at 51.6 MHz for solutions of HMM, F-actin, and acto-HMM (3 parts myosin to 1 part actin by weight). The observed k's were greater at 51.6 MHz than at 100 MHz, corresponding to the frequency dependence seen by other workers (18). Both k_1 and k_2 were less for HMM than for actin. Since k_1 for myosin A was greater than that for actin (Table I), the conclusion is that the high k_1 for myosin A is a result of a strong protein-water interaction of the tail portion of the molecule. The k_1 found for acto-HMM, 12 $\pm$ 3, was close to that calculated, 14 $\pm$ 3, using the observed values of k_1 for HMM, 12 $\pm$ 3, and actin, 20 $\pm$ 3. The corresponding k_2's were: 185 $\pm$ 20, acto-HMM measured; 135 $\pm$ 30, acto-HMM calculated; 280 $\pm$ 40, F-

actin measured; and 85 ± 15, HMM measured. Although the observed k_2 is slightly larger than that expected, the observed k_1 is slightly smaller and the conclusion is that these data indicate that no changes occur upon the complex formation. A more accurate experiment, which does not involve measurements of protein concentration or pipetting of protein solutions, is to observe the change in T_1 and T_2 when the acto-HMM complex is dissociated. Addition of ATP, 4 mM, or Mg pyrophosphate (Mg-PP_i), 4 mM, which dissociated the complex, caused no observable change in T_1 or T_2, putting an upper limit of ±10% on k_1 and ±5% on k_2 to the changes occurring in these parameters upon dissociation of the complex.

Work of Baskin and Paolini (33) on the volume changes of whole muscle showed a complex behavior with both volume increases and decreases occurring during isometric twitches. The corresponding NMR experiments have been done by Bratton et al. (20), who measured T_1 and T_2 on live muscle fibers in three states: relaxed, during isometric tension, and in rigor. They found a T_1 of 0.4 sec and a T_2 of 0.04 sec for a live relaxed muscle at 24 MHz, values which are not far from the ones we found at 51.6 MHz. They also found a 20% increase in T_2 during isometric contraction and in rigor but saw no changes in T_1. The in vitro experiments done have not revealed any protein interactions which could explain these changes.

Much work has also been done on the volume changes which occur when a suspension of glycerinated myofibrils is contracted with ATP. Baskin (34), using dilatometry, found a volume decrease or volume increase depending on the concentration of ATP which was added. Kominz (35), using an electronic sizing technique has found an increase in the protein effective volume, presumably because of an increase in hydration, to accompany contraction. We have performed an analogous experiment, measuring T_1 and T_2 via pulse methods before and after addition of ATP. Stretched glycerinated psoas fibers were used to form the fibril suspension, so that the average initial sarcomere length ranged from 2.5 to 2.7 μ. After addition of ATP these sarcomeres had contracted to 1.8–1.6 μ. As is shown in Fig. 5 no significant change is seen in T_1 or T_2 after addition of the ATP. This result is not easily related to theories of muscle contraction. The ATP added is hydrolyzed very quickly so that we are looking at a rigor muscle at two different lengths, however, the shorter of these lengths is a "supercontracted" state, i.e., since the fibrils are contracting against zero load the thin filaments have crossed through the *M* line and are overlapping each other. This is not a normal physiological state and some disruption of the filament structure (myosin-actin contacts) may have occurred. The point is that according to our results no significant change in hydration accompanies the ATP-induced contraction of a suspension of myofibrils and thus the phenomenon seen by other workers may be because of another effect.

The polymerization of actin has been considered to result in a decrease of hydration. Ikkai and Ooi (35) have shown that increased pressure favors the depolymerization of actin. T_1 and T_2 were measured for both G-actin and F-actin solutions. The value of k_1 did not change significantly when the actin polymerized; k_1 (G) = 20 ± 3,

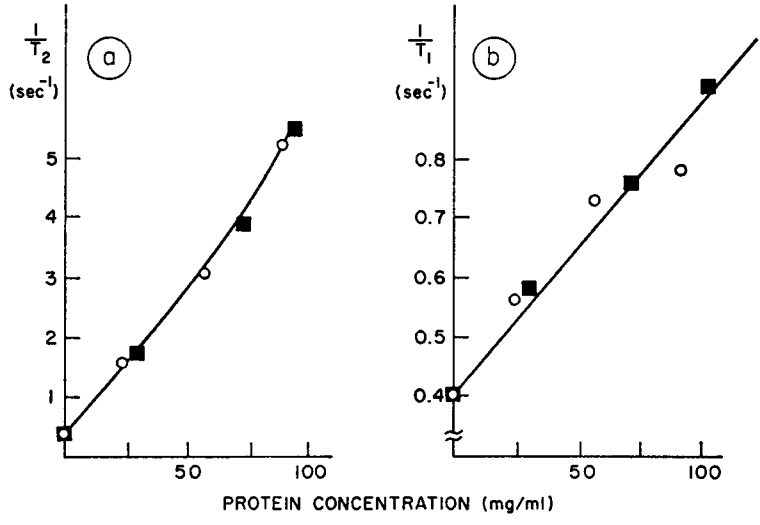

FIGURE 5 $1/T_1$ and $1/T_2$ determined by pulse-echo techniques at 51.6 MHz for water protons in a suspension of myofibrils before, ○, and after, ■, contraction by ATP.

k_1 (F) = 18 ± 3. This result was also found at 100 MHz (see Table I). In contrast, the value of T_2 decreased on polymerization giving an increase in k_2 from 180 for G-actin to 260 for F-actin.

This is the same type of behavior found by Bratton et al. (20) who observed a change in T_2 but no change in T_1 upon stimulation of a live muscle. Bratton et al. explained their data with a two-phase model in which the more tightly bound phase did not affect the longitudinal relaxation. In our model, on the other hand, the bound phase affects both T_1 and T_2 so that a change seen in T_2 due to a shift of water out of the bound phase should be accompanied by a corresponding change in T_1. There is another explanation for this behavior, however. The magnetic inhomogeneity in an F-actin solution (long rods) is much greater than in a G-actin solution (round balls) because of the shapes of the molecules. The spin-echo method will eliminate magnetic inhomogeneity only if the time that the nucleus takes to diffuse through the inhomogenous field is long compared to the time between 180° pulses.

If magnetic heterogeneity is responsible for the behavior of T_2, the observed T_2 should increase as 2τ, the time between 180° pulses, decreases. Fig. 6 shows that T_2 increases as τ decreases for both G-actin and F-actin solutions, but that the effect is much greater for the F-actin. Assuming a linear macroscopic magnetic field gradient, the contribution to T_2^{-1} due to diffusion is found to be $\frac{1}{3}\gamma^2G^2D\tau^2$ (10, p. 61), where G is the value of the field gradient, γ is the magnetogyric ratio, and D is the diffusion constant (for protons in water = 10^{-4} cm^2/sec). The protein susceptibility implies a milligauss differential in the magnetic fields surrounding the protein which, if we assume occurs in a space of 100 A, the order of magnitude of the molecular dimensions, gives a value for G of 10^3 gauss/cm. This high value for the field gradient

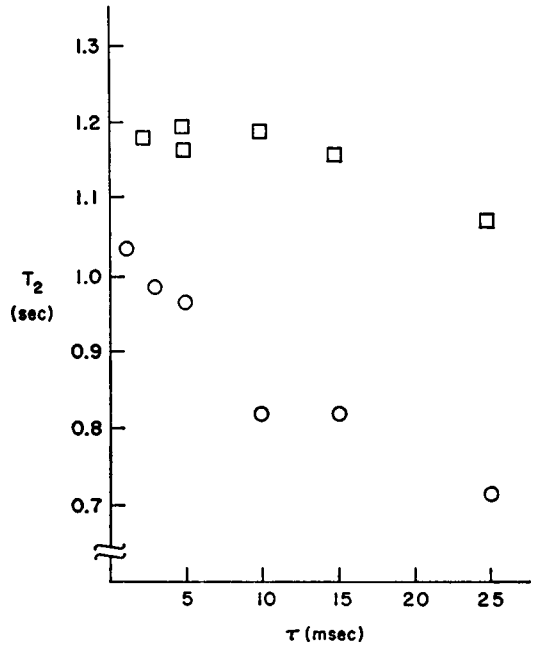

FIGURE 6 T_2 for water protons in F-actin, ○, and in G-actin, □, solutions as a function of τ, where 2τ is the spacing between 180° pulses in the spin-echo determination of T_2.

together with the large diffusion constant for the protons makes the diffusion-dependent contribution to T_2 an appreciable term even for $\tau = 1$ msec. Although the magnitude of this term is sufficient to explain the data in Fig. 6, the expected linear dependence on τ^2 is not observed; however, within 1 msec an average proton will diffuse through 10^4 A, a large distance compared to the molecular dimensions, making the assumption of a linear field gradient invalid. In this case, where the great length of the diffusion path of the nucleus tends to average over the possible values of the magnetic field, we would expect that the smaller is τ, the greater will be the increase in T_2, which is what we observe in Fig. 6. We conclude that the increased magnetic heterogeneity in the F-actin solutions explains the decreased values of T_2 and that no change in proton relaxation occurs because of changes in the bound water of the proteins when the actin polymerizes.

Aside from the change in T_1 which occurs on myosin aggregation, we have found no change in proton relaxation rates to occur for the protein interactions studied. Yet in all of these interactions other methods have given results which were interpreted in terms of hydration changes. The explanation for this discrepancy may lie in the fact that the different methods are measuring different aspects of protein hydration. Although we have shown that the interactions responsible for the increase in proton relaxation rates by proteins occur within the protein hydration sphere, we have not defined the mechanisms responsible. Until the exact mechanisms responsi-

ble for our results as well as for the results of others are delineated, it is difficult to compare them. We can say that our measurements of protein relaxation times indicate that no significant change in protein hydration occurs during the functional interactions of the muscle proteins which we have studied. This is a result to be expected if these protein interactions represent the interactions of active sites on the macromolecules whose dimensions are small compared to those of the entire molecule.

Finally we would like to end with a note of caution for those working with magnetic heterogeneous systems such as the one we have studied. The facts that the T_2 determined via pulse techniques is less than $\frac{1}{10}$ T_2^* determined from the line width, and further, that the T_2 determined by the pulse methods is dependent on the spacing of the pulses, indicate that the artifacts of heterogeneity are hard to escape. Measurements taken of heterogeneous systems are complex and great care must be taken to insure that one is measuring quantities which unambiguously give conclusions about the state of the system.

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